JOURNAL OF THE NATIONAL CANCER INSTITUTE

NATIONAL INSTITUTES OF HEALTH • U. S. PUBLIC HEALTH SERVICE

April 1950
Volume 10
Number 5

THE GROWTH OF NORMAL PLANT TISSUE *IN VITRO* AS AFFECTED BY CHEMICAL CARCINOGENS AND PLANT GROWTH SUBSTANCES. II. THE CYTOLOGY OF THE CARROT-ROOT TISSUE[1,2,3,4]

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INTRODUCTION

In the preceding paper it was pointed out that sterile fragments of the meristem of the tap root of the carrot placed on a suitable medium gave rise, in the presence of low concentrations (10^{-6}–10^{-7}) of indole acetic acid (IAA), to a nodular tissue mass. The tissue formed on the inoculum was watery in consistency and color and appeared frequently to be covered with micaceous granular bodies. After several transfers, these masses were separated from the inoculum; they increased in size by the addition of nodular masses and the whole became pigmented and opaque. Occasionally a few short roots were produced from the under surface of the mass.

Following the transfer of passage of the new growth to a medium with IAA in concentrations of (5×10^{-6}) 5 mg. per liter, the tissue mass formed was hyaline in appearance and yellow to gold in color, with many short white tapering roots.

Transfer of the nodular or hyaline tissue masses, after the medium with the higher concentration of the IAA mentioned above, to one without this growth substance, resulted in the differentiation of these cultures into complete carrot plantlets. In some instances these tissue masses differentiated even before they were transferred to an IAA-free medium. Under these conditions, poorly developed stemlike initials were formed terminated by stipule-like structures. When the tissue was transplanted to a basic medium, development ensued rapidly. New plantlets were

[1] Received July 20, 1949.

[2] Aided by a grant from the American Cancer Society, Inc. upon the recommendations of the Committee on Growth of the National Research Council.

[3] Thanks are due Dr. E. J. Baumann, Chemist, under whose direction the media in these studies were prepared.

[4] Part I. The culture of the carrot tap root meristem. Am. J. Bot. Vol. 37, 1950. Part II. The culture of sunflower and tobacco stem segments. Bull. Torrey Bot. Club 77:110, 1950.

formed that developed typical carrot leaves from the base of the stipules or the fundamental crown of the plantlet.

A third type of tissue culture was formed in which the predominating structures were thallus-like bodies. This type of tissue mass formed normal plantlets also, although the outgrowths were principally flat, pale green tongue-shaped or heart-shaped bodies. The stability of this type of culture was questioned and it appeared to be the product of the carcinogens used in these studies.

The purpose here is to report the histologic and cytologic structures of the essential tissue masses, plantlets, and thalloid structures and to interpret these findings in the light of the known effect of the chemical carcinogens on animal tissues and principally the structure of the plant tumor, better known as the crown-gall disease of plants.

The pertinent literature has been reviewed in Part I of this paper.[4]

METHODS AND MATERIALS

Cultures of the types described above were divided, grown, and studied in series of five which consisted of one grown on a basic medium (Ws) such as described by White (*1*) or one of the synthetic nutrient media used by Hildebrandt, Riker, and Duggar (*2*) for tobacco (Wt) and sunflower (Wsf) tissue. To one of these media various quantities of IAA were added to produce concentrations varying from 0.1 mg. per liter (10^{-7}) to 5 mg. per liter (5×10^{-6}). To the three others the carcinogens 1,2,5,6-dibenzanthracene (D); 3,4-benzpyrene (B) and 20-methylcholanthrene (M) were added to make concentrations varying from 1 mg. per liter (10^{-6}) to 50 mg. per liter (5×10^{-5}). Two concentrations of the carcinogens were used: 100 mg. and 190 mg. per liter, primarily to note the toxicity, if any, of these chemicals.

A basic medium of one kind was prepared by the liter and was divided into 5 parts of 200 ml. each. Only the test chemical was added to each part, and then it was divided into 25 ml. units and poured into pyrex Erlenmeyer 125 ml. flasks. Each of the 5 kinds of media consisted of 8 flasks of the same nutrients and the test chemical, 8 of basic medium, 8 of the same basic medium with IAA, 8 with D, 8 with B, and 8 with M, in a given concentration. The carcinogenic chemicals in each batch of medium were of approximately the same concentration, although a lower concentration of D was frequently used.

The tissues of related clones were divided and placed on these media and their differences in growth were studied qualitatively. The five related cultures were fixed for microscopic study for comparison, or an entire individual culture or part of a culture was fixed because it presented some morphologic uncertainty. The fixations were made in Bouin's solution as a routine procedure. Other fixatives such as prescribed by Flemming, Meves, and Carnoy were also employed. The tissues were embedded in paraffin, and sections 5 to 7½ microns in thickness were made. The staining was done according to Flemming's triple method.

Another satisfactory stain used was gentian violet counterstained with orange G in oil of cloves.

The structures of some tissues were studied by the orcein-acetic-acid smear technique for orientation and origin of tissue elements.

Photographs of the gross cultures were made shortly before fixation. Photomicrographs were made of these tissues under low power for structural studies and under high magnification for cytologic comparison.

OBSERVATIONS

The study on these tissue cultures falls into three groups: the effect of the various carcinogens and growth substances used on the tissue mass before differentiation had made its appearance, the effect of these substances on the thalloid structures, and the changes, if any, on the fully differentiated plantlets that appeared in the nodular or hyaline tissue cultures.

The nodular tissue mass.—On March 13, 1947, a series of cultures was started from the meristem of the distal end of a well developed carrot tap root. As many as 6 inocula were introduced in each of 10 flasks with White's standard medium which included 1 mg. per liter of α-naphthalene acetic acid (NAA) or 5 mg. per liter IAA. The actively growing inocula produced nodular masses of tissue. By frequent transplants and by division of the new growths a series of true tissue-culture clones was established. On July 1, clone 1_1 was grown on Ws basic standard medium, 1_2 and 1_4 were transferred to Ws with IAA; 4_1 on Ws and 4_2 on Ws IAA; 4_3 on WsD 0.5 mg. per liter; 4_4 on WsB 1 mg. per liter and 5_1 on WsM 1 mg. per liter; and 5_2 on Ws IAA. These clones are shown in figures 1*A* and 1*B* as photographed on July 18, 1947.

Fixations were made from these tissues on August 11, 1947, and on October 2, 1947. Clone 5_2 was divided on August 11 and figure 2 represents a low-power view of a section taken through the upper part of the nodular tissue mass grown on a standard medium. The effect of the IAA, upon which it had been grown prior to the date of transfer, seems no longer evident in the serial sections made of the new growth of this tissue. Only the peripheral cells which show evidence of elongation suggest an IAA effect.

In this section, two central nodular masses may be discerned. The cells are of irregular size and shape. The cytoplasm stains faintly, and the nuclei are small and not well differentiated. This may be attributable to the lapse of time between the last transfer and the period of fixation.

The portion of the 5_2 clone that was transferred to IAA gives evidence of morphologic differentiation, and the effect may be ascribed to the indole acetic acid. The loose peripheral cells were elongated and contained little cytoplasm, which was distributed along the cell walls. The cells were large, and the small nuclei occurred in section only on occasion. The interior cells were like those grown on standard media.

There were suggestions of division of the cytoplasmic mass within the walls of the old cells, as indicated by Gautheret (*3*) in the new growths of

carrot segments. A more detailed study of this phenomenon is reported below. In some sections new nodules made their appearance. Here fibrovascular elements have developed. These were disoriented and lay in the middle of a group of active cells with fine granular cytoplasm and with well differentiated though faintly staining nuclei, as shown in figure 3.

The vessels were not attached to any well developed fibrovascular system, for similar nodules fixed *in toto* and stained by the orcein-acetic-acid smear technique showed them to be independent isolated entities with annular or scalariform markings. This type of tissue is like that found in sections of crown-gall tissue induced by *Phytomonas tumefaciens*.

The presence of these isolated vessels has been the criterion upon which overgrowths on plants have been diagnosed as crown gall. That these elements in the tissue mass are formed *de novo* cannot be questioned. What role IAA plays in the development of crown gall is not yet clear. This does not support the contention that plant overgrowths or plant tumors are solely the products of IAA. Other workers such as Kraus, Brown, and Hamner (*4*), Hamner and Kraus (*5*), Link, Wilcox, and Link (*6*), Brown and Gardner (*7*), and Berthelot and Amoureux (*8*) contend that the factors responsible for plant tumor growth are induced by the presence of indole acetic acid. However, the studies of Camus and Gautheret (*9*) tend to show that crown gall differs physiologically from overgrowths induced by this growth substance. Confirmation of these observations is of importance, for a comparative study of the histologic preparations of crown-gall tissue and tissue grown *in vitro* subjected to IAA shows that they are not very different. The appearance of what has been considered diagnostic traits, such as the presence of isolated fibrovascular elements, makes them indistinguishable in sectioned material.

However, another distinguishing characteristic feature of crown-gall tissue is worth repeating, for in this tissue young embryonic cells form its boundary, and growth is peripheral. In this respect crown gall behaves like an animal neoplasm. In the tissue cultures grown on IAA the surface cells are large, the cytoplasm is sparse, and the nuclei are disproportionately small in comparison with the size of the cell. The elongated cells generally arise on the periphery of the tissue. In the crown gall, long cells such as these may represent protoxylem cells; these have a more abundant cytoplasm, well differentiated nuclei, and occur in the interior of the tissue mass.

In clone 5_x–2 (fig. 4) fixed on October 2, 1947, from a culture that was last transferred to Ws with IAA on August 11, 1947, a typical IAA reaction is shown. The nodular tissue masses have given rise to a number of roots. Here differentiation of the tissue has advanced to the stage of organ formation. These roots appear to arise from small clusters of small cells that bear resemblance to cells that constitute the pericycle. The tissue mass now presents the appearance of a stem treated *in situ* with IAA. The cells that constitute the tissue mass are like those seen

in tissue grown on basic medium, noted above in the early stage of development. Here too are evidences of cellular dissociation about the periphery of the nodular masses and at the bases of the emerging roots.

The clone 3_3 grown on medium Ws with D 0.5 mg. per liter and fixed on August 11, 1947, 6 weeks after the last transplant, showed the nodular mass with three nodules forming a Y-shaped structure with a portion of a fourth mass in between the two upper arms of the Y. The outer cells of the whole tissue mass are large, loose, and disoriented. On the periphery, small groups of active cells with dense cytoplasm and small nuclei having the appearance of embryonic tissue are seen. These cells form a surface layer two or three cells thick and have a tendency to organize as an active meristematic tissue. The central tissue mass is composed of cells of variable size. The cytoplasm forms a thin layer about the cell wall as noted in previous section of growths on basic medium with IAA; the nuclei are small and inconspicuous.

From portions of the same tissue mass of clone 3_3 several sections revealed more advanced stages of cellular differentiation and organization. In figure 5 a part of a nodule is shown. The old cells at the left and above the actively growing cells of the nodule are seen. The organization consists of an imperfectly formed thin layer of cells surrounding the new tissue. These cells are small, with well differentiated nuclei, and give the appearance of an epidermis in the process of development. The cells below this layer are of a large parenchymatous type with cytoplasm that has become partially plasmolyzed, possibly due to the fixing agent. The nuclei are well differentiated and apparently normal; division figures are not observed. Fibrovascular elements appear as isolated, densely-staining bodies but their presence is not so common as in the IAA-treated tissues. Here it appears that the D has stimulated centers of growth and organization, apparently consistent with the early differentiation of the tissue mass to form miniature carrot plants. In these D-treated tissue masses root initials also were formed.

Cultures in this series treated with B or M showed growth stimulation expressed in isolated centers that resolved themselves into small tissue masses resembling strands in cross-section. These occurred on the periphery of the old parts of the tissue mass or in the center of well developed nodules. The cells were active, and differentiation of tissue formed isolated groups of marked vessels as shown for the IAA-treated tissue in figure 3. This new growth was characterized by dividing cells. The cells were vacuolated, with well differentiated nuclei. The granular cytoplasm was gathered about the periphery enveloping the laterally placed nuclei.

In division phases shown in figure 6, the small chromosomes typical of the species are observed at the poles in early telophase with the new cell plate in process of development. This photograph represents a tissue mass grown on 3, 4-benzpyrene. With various concentrations of the carcinogens the thalloid type of tissue masses developed. The influence of the carcinogens on these bodies is reported below.

The concentrations of the carcinogens used on the nodular tissue mass of the carrot indicate a stimulative effect on certain cells or groups of cells which give rise to new centers of activity. Differentiation occurs, and independent fibrovascular elements are formed which play no role in the biologic economy of the tissue mass. The same type of cellular differentiation is observed after treatment with IAA and the carcinogens. Evidence of transformation of normal cells in these tissue masses into aberrant, atypical neoplastic elements has not been established.

Abortive thalloid structure.—Testing the effect of higher concentrations of the carcinogens was undertaken on a culture 7,222 that had its origin in April 1946. On October 30, 1947, it had been on a basic medium with IAA (5 mg. per liter) for the previous three passages, and on this date it was divided and cultures on D, B, and M in concentrations of 30 mg. per liter were established. These cultures are shown in figure 7, photographed on December 22, 1947. The first flask contains a subculture of the clone on Wt and the second contains the same medium with 5 mg. per liter IAA; the other three cultures were grown on the same basic medium with the addition of D, B, and M at a concentration noted above. In D, B, and M cultures, roots were well developed and the tissue masses showed evidence of abortive thallus-like initials. On December 29, 1947, these cultures were divided again, and the active tissue was transferred to a similar basic medium to which IAA 5 mg. per liter was added to one series and the carcinogens had been increased to 100 or 190 mg. per liter. Figure 8 shows a series of cultures with D, B, and M at 190 mg. per liter on January 23, 1948. The thalloid processes increased in B and M in spite of the high concentrations of these carcinogens. Observations on cultures grown on D have shown this agent to be more active than B or M. Here the growth had not progressed although the tissue appeared viable. Photographs of these cultures made a month later failed to show measurable increases in the tissue masses.

Sections of the typical tissue masses of the cultures grown on D, B, and M in concentration of 100 mg. per liter appeared to differ little in gross appearance. Flattened irregular-shaped masses of tissue arose from the transplants in each case. The cross section of the tissue mass grown on M, however, showed some proliferation of the epidermal cells. These consisted of embryonic cells crudely organized, and differentiated only by the formation of a distinct epidermal coat. The internal cells were small, with large nuclei, and normal division figures were not infrequent.

Clones of 7,225 derived from a division of a culture grown on M on November 3, 1947, yielded four subcultures of which one culture was grown on Wt. Subculture 2 was grown on Wt plus IAA, and cultures 3 and 4 were grown on B 16 mg. per liter and M 22 mg. per liter respectively. The cultures grown on Wt and WtB showed a tendency to form thalloid structures whereas the M culture increased markedly in size and evidence of pronounced thallus-like bodies appeared (fig. 9*A*). Growth of the tissue mass on the Wt IAA was nodular.

On December 29, 1947, the carcinogen-treated cultures were transferred to carcinogens of higher concentration (190 mg. per liter). On February 10, 1948, the control and the M-treated culture showed pronounced abortive, thallus-like bodies (fig. 9*B*). The IAA and B cultures remained unchanged; sections of the material showed a control similar to that of the M-treated culture. The latter, however, produced fringes of new nodular tissue from the margins of the thalli similar to those seen in cultures with concentration of the carcinogen at the level of 100 mg. per liter.

An attempt to determine the fate of these bodies was made. Following three subdivisions of the normal cultures mentioned above to basic media and to media with D, B, and M in low concentrations, a diminution of the abortive thalloid tendency and a return to the nodular type of culture were produced.

Another series of clones that presented suitable material for cytologic studies were 7_1x7_1, 7_1x5_4, and 7_1x10_1. These were derived by division of a common stock culture on February 13, 1947. Clone 7_1x7_1 was divided on May 14, 1947, and its fragments were grown on the Wt medium; Wt IAA 5 mg. per liter; WtD 0.7 mg. per liter; WtB and WtM, each at a concentration of 1.2 mg. per liter. On July 18, 1947, the D, B, and M cultures showed thalloid structures. On August 9, 1947, all the cultures were transferred to fresh basic medium Wt, and on September 17, 1947, the tissue mass in the control culture was pigmented. Those cultures that had previously been grown on media with a carcinogen produced several feeble green and white plantlets.

On November 25, 1947, the normal, IAA and D cultures were divided into five or more parts each, and were seeded on the five different kinds of media used in these experiments. In these cultures, a concentration of 20 mg. per liter of D, B, and M was used. A part of a section of an abortive thalloid structure is shown in figure 10*A*. This culture was grown on Wt medium with 20 mg. per liter of D. The main body shows a mass of parenchymatous cells with small nuclei and a moderate scattering of pigment bodies. Giant cells are sparsely scattered throughout the tissue. Two are to be seen near the center of the figure. In these cells the nuclei are well differentiated, the nucleoli stain a ruby red, the chromatin granules stain purple violet, and the reticulate cytoplasm stains faint orange with Flemming's triple stain. In this section a collection of disoriented vessels with their characteristic markings is seen. Reference to this type of cellular organization has been made above and elsewhere *(10)*.

The peripheral cells of these abortive thalloid structures give evidence of meristematic activity. Giant cells seen near the margin or in the interior of the body have large vacuoles with small nuclei. Occasionally the nucleus in these cells divides repeatedly, forming a mass of cells within the confines of the old cell wall (fig. 10*B*). This photograph was made from the same thallus-like body, a part of which is shown in figure 10*A*. The large, single cell with its nucleus lying in the sparse cytoplasm is bound by a heavily lignified cell wall. The cell below shows a similar cell wall, but its cytoplasmic and nuclear structures have grown and been

divided to make new cells that fill the cavity made by the old wall. This is not an unusual occurrence in these tissues. The contents of the cells of the surface meristem and certain interior cells frequently divided in these thallus structures within the space formed by the mother wall. Gautheret (*3*) figured this type of division in primary outgrowths from carrot segments. This type of division appeared frequently among the giant cells in the carcinogen-treated tissues; the epidermal meristem in the untreated tissue proliferates less actively in this fashion.

Culture $7_1 7_3$ was grown on D on a Wt medium from May 14, 1947, to March 1, 1948, during which period it was returned to a basic medium once. On the latter date it was again returned to WtD in concentration of 100 mg. per liter. The subculture studied showed changes that were worthy of note. These consisted of a greenish tissue mass with abortive thallus-like bodies and some green leaves that were lying in the medium. This material was fixed on April 16, 1948, and a low-power view of a section of the leaflike structure is shown in figure 11*A*. One margin of this structure shows a nodular mass of hypertrophied cells. The cross-sections of the vascular bundles represent normal veins, except two. These are composed of parenchymatous cells with tracheids resembling the organization of crown-gall tissue. The lower surface of this structure as seen in the photograph is bound by a double layer of epidermal cells. The presence of stomata was not detected although normal leaves in these cultures showed perfectly formed guard cells and stomata. One of these proliferating regions was photographed under higher magnification and is shown in figure 11*B*.

The tumor-like tissue consists of a mass of parenchymatous cells with part of its epidermis eroded. The cells appear normal, and well differentiated nuclei that respond typically to Flemming's triple stain are found. The peripheral cells are small whereas the interior cells are large. The nuclei in both regions vary slightly in size. Lying in the parenchyma are cells with thickened reticulate or annular markings. In this instance leaflike structures grown on dibenzanthracene show histologic and cytologic characteristic overgrowths not unlike those observed in plant tumors induced by bacteria. The response of the plant tissue to a suitable chemical stimulus differs little morphologically from its response to the parasite *P. tumefaciens*. Crown gall or plant tumor represents a protective reaction to the bacterial agent. The reactions to these chemicals seem similar and they are alike in function.

Clone $7_1 \times 5_4$ had an origin similar to that of $7_1 \times 7_1$ cited above. The gross appearance of the subcultures of this clone grown on Wsf, Wsf IAA 5 mg. per liter, WsfD 35 mg. per liter, WsfB 50 mg. per liter, and WsfM 50 mg. per liter, on January 27, 1948, is shown in figure 12*A*. The last subculture was made on December 8, 1947. The D, B, and M treated cultures consisted of thickened protuberances arising from a nodular tissue mass. Sections of the growth from the D culture gave evidence of many hypertrophied giant cells in the center of the growth. Figure 12*B* represents a part of the parenchymatous tissue. The centrally placed cell

in this photograph shows a daughter nucleus that appears to have divided twice without cytoplasmic partition. The other daughter nucleus in the other daughter cell has moved and does not appear in this section. There is evidence to show that only one daughter nucleus of a giant cell may divide several times without accompanying cell division. This aberrant cytologic phenomenon may be the result of the chemical carcinogen employed in the medium.

Sections of the subculture grown on the medium with M present a parenchyma with large, elongated cells. Atypical, spirally-marked vessels in distorted arrangements are common. Nucleate giant cells in division phases have not been observed in this tissue.

The B-treated culture in this series, grown only from December 8, 1947, to January 28, 1948, showed hypertrophied cells with small, inactive, poorly staining nuclei. In the epidermis of these bodies hyperplasia of the surface meristem occurred. Here also the old cell walls retained the nuclear divisions until the lumen of the cell was filled with new cells.

Another culture, $1_2 4$, grown on Wt with benzpyrene at a concentration of 50 mg. per liter, was carried through a number of subcultures. The last transfer was made to Wsf medium with B, in a similar concentration of the carcinogen on December 11, 1947. This culture in the gross consisted of flattened, pale-green bodies, and 39 days after the last transfer it was fixed for microscopic examination. A section of this material is shown in figure 13. The body consisted of parenchymatous tissue with large irregular shaped cells. A distinct epidermal layer is absent although remnants of a degenerated structure appears evident. At a number of points along the body, cells have proliferated to form irregular masses of embryonic tissue. Where proliferation failed, a thin heavily stained band of meristematic tissue represents the remains of what might have been epidermal cells. Here, also, old cell walls form the boundaries of a number of new cells that have developed within their cavities. Frequently these masses of cells represent the activity of a single cell. A study of these embryonic masses reveals them to be the products of division of a number of adjacent epidermal cells. The body mass shows traces of oriented vascular elements although their size and number appear to be small.

The thalloid structure.—A number of complete series of cultures with thallus-like bodies that arose from the hyaline tissue mass were studied microscopically. One of these is presented in culture $7_1 x10$ described in the gross in Part I of this report[4]. The last division and transfer were made on April 28, 1947, and fixed on July 7 of the same year. This series was grown on Ws as control, with Ws and IAA 5 mg. per liter, and the carcinogens in concentrations of 0.5 mg. per liter for the D and 1 mg. per liter of the B and M constituted the other culture media of this group.

Section of the control culture, $7_1 x10_1$, reveals some irregular shaped bodies with a number of isolated, new organoids arising from its margins. The body consists of an irregular shaped, thallus-like structure composed

of elongated, disoriented cells of variable size. Vascular elements appear in the old part of the tissue. The epidermis is thin, showing a number of globular masses of tissue consisting of parenchymatous and small lignified cells. The normal growth proceeds at several regions of the body, giving rise to irregularities in the contour of this structure.

A small portion of this thallus-like body is shown in figure 14. The proliferation of the epidermis resulted in the mass to the right. Its differentiation to form an epidermal covering is incomplete. Its parenchymatous cells are large, and only a few of them appear to have nuclei. Above this globular mass a part of the body of the thallus is shown, in which the growth activity is directed against the old tissue, causing compression of the newly formed cells. The cytoplasm in these cells is abundant, and its granular inclusions obscure the small nuclei. This culture represents the behavior of these thalloid bodies on standard media. Growth is not confined to an apical meristem but to meristematic cells on the surface of the body. Differentiation appears normal in cross and longitudinal sections.

In the IAA-treated culture in this series, evidence of dissociation is pronounced and some collapsed cells are present. Yet organoids appear to arise from the epidermis, and small clusters of cells in that tissue seem to have arisen within the old cell boundaries. The cells have characteristically stained nuclei and appear active. These organoids are composed of cells with coarse granular cytoplasm in which a moderate dispersal of pigment bodies is found. In parts of the new growth some nuclei appear pyknotic; in others, the cells are large and the cytoplasm is finely granular; and in still other parts, the cells are small with dense cytoplasm.

The culture grown on media with D in this series produced thallus bodies composed of hypertrophied parenchymatous cells surrounding a disoriented mass of fibrovascular elements. The surface layers of these bodies were lined by small, active, and proliferative cells, many of which were pyknotic. Many new organoids arose from this active surface layer. The epidermal cells in these new structures have also undergone intensive proliferation. The cells are small, and their nuclei comparatively large. This tissue is not unlike that observed in other tissue masses cultured under similar conditions. The vascular elements and the intense proliferating surface meristem seem to be associated with the effect of the carcinogen, dibenzanthracene.

Cultures grown on a medium which included B or M in this series also showed extensive proliferations of the epidermal structure in these thallus-like growths. Figure 15 is a low-power view of a section of a thallus body grown on benzpyrene. These thalloid bodies were flattened structures with the entire surface meristem in an active, proliferative state. The body was composed of parenchymatous tissue with few fibrovascular strands. The cells lining a lumen frequently showed meristematic activity. The methylcholanthrene-treated tissues in this series appeared very much older. The B-and-M-treated cultures had fewer proliferations

from the epidermal growth than were seen on similar bodies grown on media with D.

The tissue mass of the thallus type grown on D showed greater activity, and this appears to be attributable to the influence of this carcinogen. Tissue differentiation too appeared more pronounced in these carcinogen-treated cultures. The evidence seems clear that lower concentrations of the carcinogens used on these cultures served as stimuli to cell multiplication.

The transformation of these cells into neoplastic tissue cannot at present be affirmed. The fact that in the production of plant overgrowths a similar process ensues is of interest. The introduction of *P. tumefaciens* stimulates cell proliferation. These initial stages and those in which the histologically characteristic structures of the crown gall are stimulated, seem to be attained through the effects of the carcinogens incorporated in the medium on which these thalloid structures developed. The control thalloid specimens showed growth of the surface meristem, but those grown on the D, B, or M resembled histopathologic entities not unlike the crown-gall disease. Dibenzanthracene seemed more active than the other carcinogens used in these studies.

The carrot plantlet.—The effect of the carcinogens on the tissue mass and the thallus-like type of growth suggested the possibility of some structural changes in the plantlets that arose in these cultures. Gross-examination of these plantlets frequently showed minute nodular masses on the part of the plant from which the leaves arose. This structure becomes transformed into the tap root. Yet in these cultures with a shallow supply of medium it most frequently developed above the substrate or lay prostrate in it. When these plantlets were separated from the tissue mass and removed to large test tubes with double the quantity of medium used in the flasks, they grew and produced an abundance of leaves, all arising from a common region between the stipules, and the region below thickened to form a miniature tap root. These tap roots were of uniform diameter, but occasionally typically shaped and colored miniature carrot roots were formed.

A culture started from clone 6_1 on June 10, 1946, was divided and set out on August 8, 1947, on Wt medium: Wt with IAA 5 mg. per liter, WtD 3.5 mg. per liter, WtB 5 mg. per liter, and WtM 5 mg. per liter. All cultures except the one grown on IAA formed complete plantlets. The IAA, while it retarded the growth of the plantlets, did not destroy the initials, for when this culture was subcultured to a medium without IAA or to a lower concentration of it or to media with D, B, or M, plantlets arose in abundance.

Plantlets derived from the B culture were grown on the different media at the same concentration levels used in the studies mentioned above. These were removed from the media on January 2, 1948, photographed, and then fixed. The plantlets shown in figure 16 were grown on (6_14_1) normal substrate, (6_14_4) B medium, and (6_14_5) M medium. Sections of these plantlets were made. Plantlet 6_14_1 in section revealed a bizarre,

cellular structure not unlike that observed in well developed plant tumors resulting from inoculation with *P. tumefaciens*.

The longitudinal section (fig. 17) of this aberrant tap root shows an enveloping cortical layer on the upper part and its erosion and destruction on the lower surfaces. The area in between is occupied by a tumor-like tissue mass not unlike crown-gall tissue in appearance. Old tissue is seen on the upper and lower left surfaces. The tissue of the new growth consists of whorls of embryonic cells in which lignified vascular elements are prominent. These whorls are not uniform but are distorted as shown in the lower right.

Here, too, the lignified cells are prominent. Such figures are suggestive of crown gall in appearance. This view is further confirmed by a study of this tissue under higher magnification as shown in figures 18*A* and 18*B* taken from sections adjacent to the one shown in figure 17. The cells are variable in size and stain differentially with Flemming's triple stain. Division figures occur frequently but the size and number of the chromosomes give no indication of polyploidy encountered in mature portions of crown-gall tissue (*10*).

All plantlets grown on the carcinogens did not respond in the same fashion to these agents. Many of them developed large hyperplastic growths at the tip of the tap root consisting of parenchymatous cells only.

Crown gall on tissue-culture plantlets.—Crown galls have been produced on the elongated tap roots of a number of plantlets without contaminating the medium which had fostered their growth. The plantlet shown in figure 19*A* came from a culture on basic medium, and the inoculation was made with a virulent strain of *P. tumefaciens* 15 days prior to the photograph. These plantlets were sectioned and studied. Figure 19*B* is presented for purposes of comparison. The plantlet selected was well grown and carried an overgrowth 1 mm. in diameter. The histologic structure was very similar to that shown in figure 17. Here again is shown an embryonic leaflet that seems to have arisen from the crown-gall tissue. The cellular structure of the crown gall is now well known and needs no further description.

The presence of these tumor-like structures in the tap roots of plantlets grown under controlled conditions on media with a carcinogen does not imply that the chemical produces crown galls. It does, however, indicate that plant tissues under the influence of stimuli that come from a bacterial parasite like *P. tumefaciens* or from the chemical carcinogens used here react similarly. The assumption that these cells are transformed neoplastic plant cells with autonomous growth and with many of the attributes of animal cancer cells is not indicated. Although these two types of tissue, crown-gall and carcinogen-treated tissue, are morphologically similar, their physiologic properties are to be investigated.

These carcinogen-treated tissues were not grafted on plants grown *in situ*. It would appear that such a procedure would help to determine the presence of an increased stimulus to growth in this tissue. Studies of plants treated *in situ* with chemical carcinogens indicated scarlike reactions

that resemble crown-gall tissue but never attain the proportions of an actively proliferating localized mass. Carrot tissue grown *in vitro* is embryonic in its physiologic and morphologic potentialities and may live indefinitely (*3*, *11*).

Structural changes in the plant tissue that resemble crown gall can be induced in this tissue by the addition of indole acetic acid or the chemical carcinogens to the nutritive substrate. Its transformation into a malignant growth such as occurs in animal experimentation is questioned.

When the mechanism of gall formation is discovered, it is probable that the mechanism for the initiation and development of neoplastic growth in animals may be better understood.

These studies show that the carcinogens induce cellular changes in plants that resemble morphologically the neoformations induced by inoculating a susceptible host with *P. tumefaciens*. The intensity of the reactions is limited in both cases by the nature of the host.

SUMMARY AND CONCLUSIONS

The nodular tissue mass derived from the meristem of the carrot tap root presents an inverted cup-shaped structure made up of parenchymatous cells of variable size with small, poorly staining nuclei when grown on basic media with low concentrations of indole acetic acid (IAA).

These masses grown on media with addition of IAA 5 mg. per liter gave rise to tissue masses that included cells with lignified walls and spiral and annular markings. These cultures grown on IAA developed amorphous, hyaline, orange-colored masses with abundance of roots, and in subsequent transfers to basic media or media with a chemical carcinogen gave rise to completely differentiated carrot plantlets.

Introduction of these tissue masses to media in which the chemical carcinogen 1,2,5,6-dibenzanthracene (D), or 3,4-benzpyrene (B) or 20-methylcholanthrene (M) were dissolved and suspended showed tendencies of the masses to proliferate peripherally and to produce a moderate occurrence of fibrovascular elements in the mass to make this tissue resemble crown gall or the IAA-treated tissue.

Differentiation of the hyaline or nodular tissue mass gave rise to a third type of tissue culture that consisted of thallus-like bodies. These studies showed these structures to be composed of parenchymatous cells with some strands of tracheids, and the entire body was bound by an epidermal meristem. Proliferation from this surface meristem resulted in the formation of rudimentary masses of embryonic tissue. These appendages on the thalloid body appeared more abundant in the carcinogen-treated cultures. Nuclear divisions were frequent in these proliferating outgrowths. It seems that the tendency of these thallus bodies to produce peripheral outgrowths was enhanced by the presence of carcinogens. It also seems that D induced more active reactions than B or M. Giant and normal cells appeared in these cultures and showed evidence of nuclear and cell division within the confines of the walls of the old mother cell. A single cell may give rise to a new body. This demonstrates the possibility of a single cell giving rise to a new growth, benign or malignant.

Plantlets grown on the carcinogens showed aberrant cellular formations that suggested crown-gall tissue. Cell and nuclear divisions were of the normal type and occurred frequently.

It is concluded that stimuli arising from the pathogen *P. tumefaciens* or the chemical carcinogens bring about similar cellular changes in the plant. It indicates the limited capacity in which the plant organism reacts to a variety of different and apparently unrelated substances.

The structural changes induced by the carcinogens in the *in vitro* cultures of carrot meristem are not malignant, and they are not unlike the plant tumors produced by the bacterial parasite.

The capacity of these tissues, normal as well as those carcinogenically treated at extremely high levels of concentration, to perpetuate themselves through a period of three years suggests the need for some simple device such as a technique to enable one to separate crown-gall tissue from those tissue responses induced by the chemical carcinogens. It is probable that the isolation of the factor that is supplied by the parasite and/or host responsible for cell proliferation, may throw light on this and other related problems associated with neoplastic growth.

The tissue culture method of study appears justified because primarily the influence is exerted directly on the tissue without the intervention of extraneous factors known and unknown that besiege the plant tissue *in situ*.

REFERENCES

(*1*) White, P. R.: Handbook of plant tissue culture. pp. 277. J. Cottell Press (1943).

(*2*) Hildebrandt, A. C., Riker, A. J., and Duggar, B. M.: Influence of composition of medium on growth in vitro of excised tobacco and sunflower tissue cultures. Am. J. Bot. 33: 591–596 (1946).

(*3*) Gautheret, R. J.: La culture des tissus. pp. 202. Gallimard, Paris (1945).

(*4*) Kraus, E. J., Brown, N. A., and Hamner, K. C.: Histological reactions of bean plants to indoleacetic acid. Bot. Gaz. 98: 370 (1936).

(*5*) Hamner, K. C., and Kraus, E. J.: Histological reactions of bean plants to growth promoting substances. Bot. Gaz. 98: 735 (1937).

(*6*) Link, G. K. K., Wilcox, H. W., and Link, A. DeS.: Responses of bean and tomato to *Phytomonas tumefaciens*, *P. tumefaciens* extracts, indoleacetic acid, and wounding. Bot. Gaz. 98: 816 (1937).

(*7*) Brown, N. A., and Gardner, F. E.: Indoleacetic acid galls of a secondary type. Phytopath. 27: 1110 (1937).

(*8*) Berthelot, A., and Amoureux, G.: Sur la formation d'acide indol-3-acetique dans l'action de *Bacterium tumefaciens* sur le tryptophane. Compt. Rend. Acad. Sci. 206: 537 (1938).

(*9*) Camus, G., and Gautheret, R. J.: Nouvelles recherches sur le greffage de tissus normaux et tumoraux sur des fragments de racines de Scorsonère cultivés *in vitro*. Compt. Rend. Soc. Biol. 142: 769 (1948).

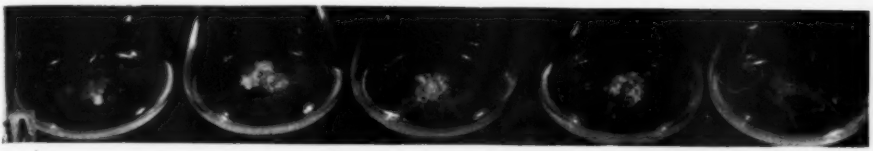
(*10*) Levine, M.: Studies in the cytology of cancer; crown gall disease; historical development. Am. J. Cancer 15: 1410–1494 (1931).

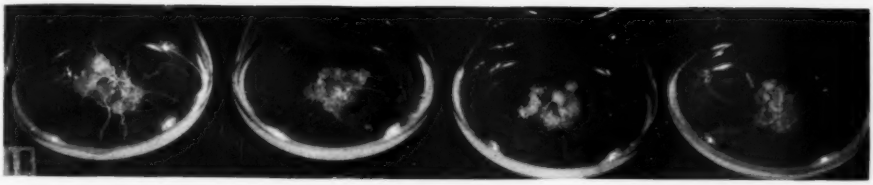
(*11*) Nobécourt, P.: Cultures en serie de tissus végétaux sur miliu artificiel. Compt. Rend. Acad. Sci. 205: 521 (1937).

PLATE 148

FIGURE 1.—*A*, Left to right clone 1_1 Ws: 5_2 Ws plus IAA 5 mg. per liter; 4_3 Ws plus D 0.5 mg. per liter; 4_4 Ws plus B 1 mg. per liter; 5_1 Ws plus M 1 mg. per liter. Note the thick fuzzy roots in 4_3 and 5_1, the nodular mass in the control with few fibrous roots, the hyaline mass in 5_2. × ½. *B*, Left to right: clone 4_1 on Ws; clones 1_2–1_4–4_2 on Ws plus IAA 5 mg. per liter. Note the uniformity in development of the three clones on IAA, also the fuzzy roots on 4_2. × ⅔.

FIGURE 2.—Section through the upper portion of nodular tissue mass (clone 5_2–3) grown on Ws basic medium: last passage August 11, 1947, fixed on October 2, 1947. Cells in the center are large, nuclei small and stain faintly, and cytoplasm is sparse. Some peripheral cells are elongated. × 35.





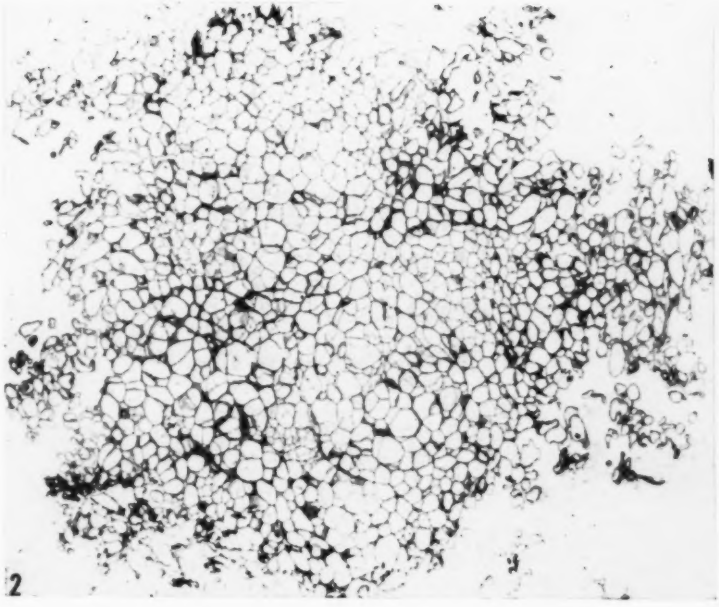
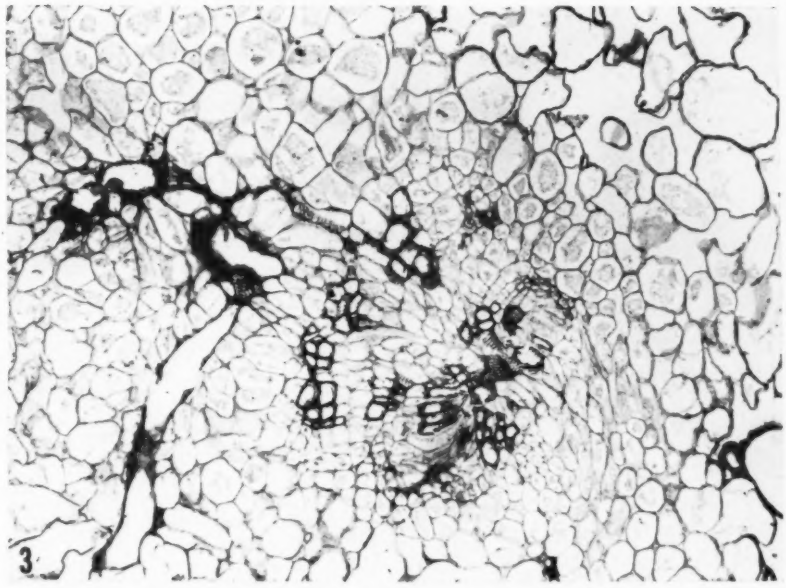


PLATE 149

FIGURE 3.—Portion of tissue grown on IAA showing differentiation of fibrovascular elements suggestive of crown gall or plant tumor tissue. × 100.

FIGURE 4.—Cross-section of tissue mass grown on basic medium with IAA 5 mg. per liter. Note the new roots. Also note dissociation of cells at the bases of emerging roots. × 35.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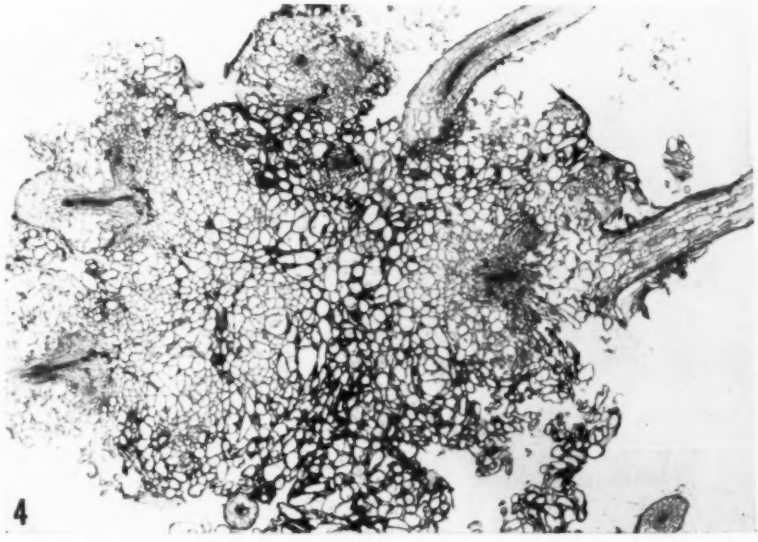
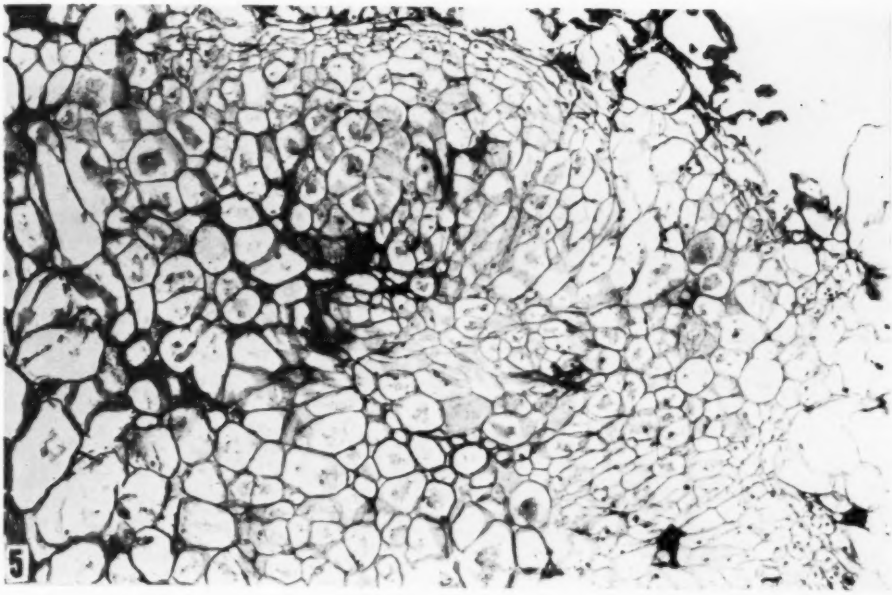

4

PLATE 150

FIGURE 5.—A part of clone 3_3 tissue mass grown on Ws with 1,2,5,6-dibenzanthracene (D) 0.5 mg. per liter for 6 weeks showing parenchymatous cells surrounded by a partially enveloping epidermis. × 100.

FIGURE 6.—Clone 3_4 grown on basic medium with 3,4-benzpyrene (B) showing normal nuclear division in early telophase with cell plate in process of development. × 1,125.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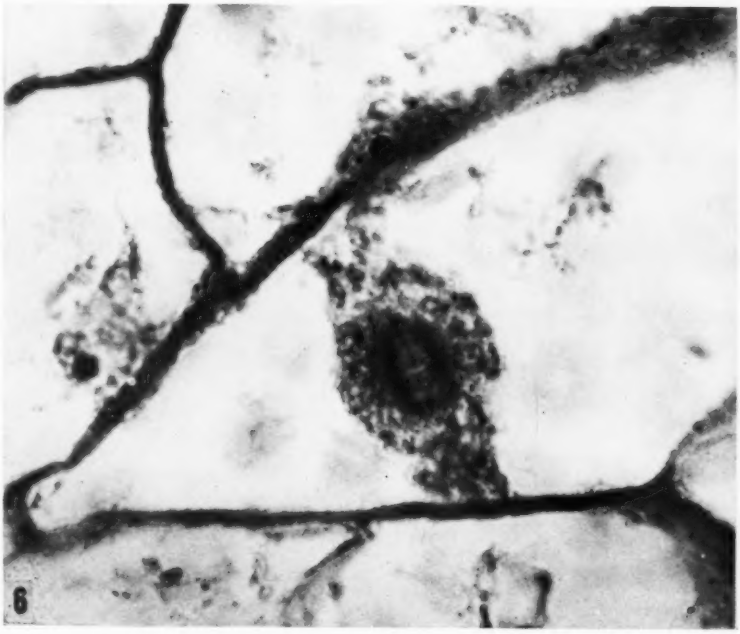
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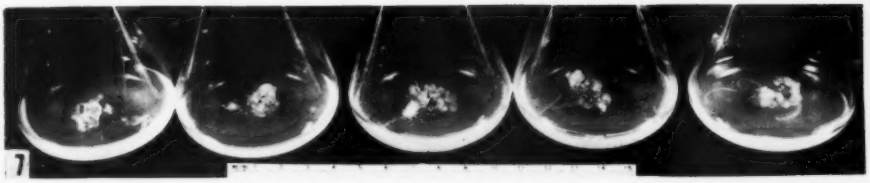
PLATE 151

FIGURE 7.—A series of test cultures, read from left to right (Wt basic medium used in all); the first Wt; Wt IAA 5 mg. per liter; WtD; WtB; WtM, in concentrations of 30 mg. per liter each. Thallus-like initials are appearing as isolated white patches on the nodular masses. × ½.

FIGURE 8.—Subcultures of the 7_1222 series transferred to flasks with Wt medium but the carcinogens are now at a level of 190 mg. per liter. Note the development of abortive structures in B and M. × ½.

FIGURE 9.—*A*, Four clones of 7_1225 that had been grown on a high concentration of M. From left to right on Wt; basic medium IAA; B 16 mg. per liter, and M 22 mg. per liter. Thallus-like bodies are evident in all except the IAA grown culture, 49 days after the transplant. × ⅔. *B*, Transfers of these cultures to higher concentrations of B and M. Control and IAA first and second figures from left. Note abortive thallus-like bodies in control and in M cultures. × ½.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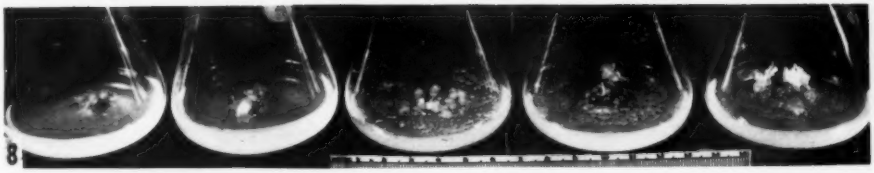
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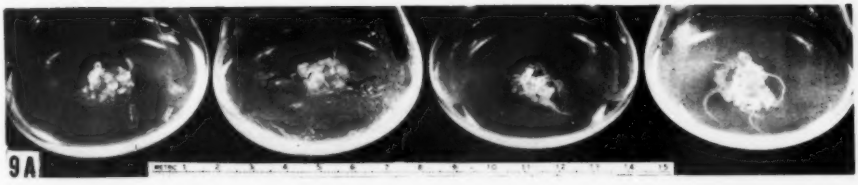
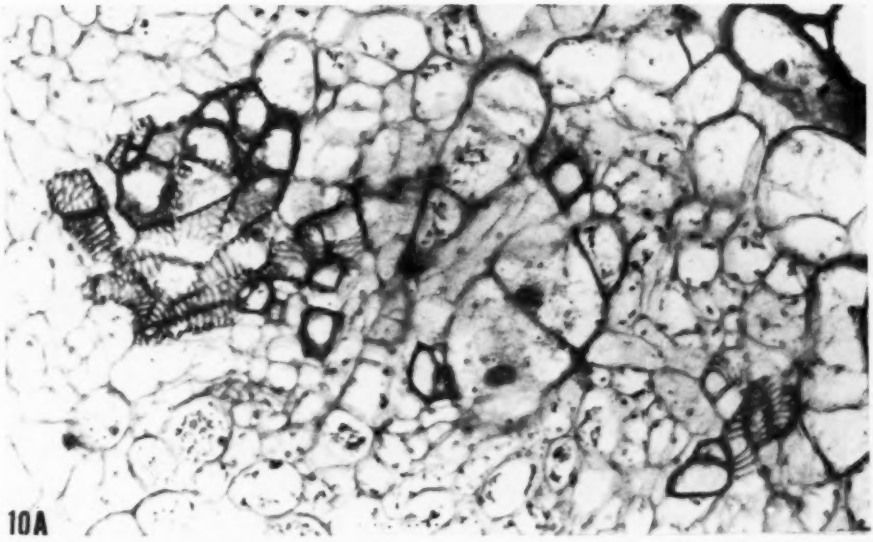

9A

9B

PLATE 152

FIGURE 10.—*A*, Section through thallus-like body grown on Wt plus D 20 mg. per liter, with giant cells and lignified vessels. × 260. *B*, Giant cell the contents of which divided to form a number of cells. Note the giant cell with a large vacuole and a small, well differentiated nucleus in the same field. From the same tissue as shown in *A*. × 520.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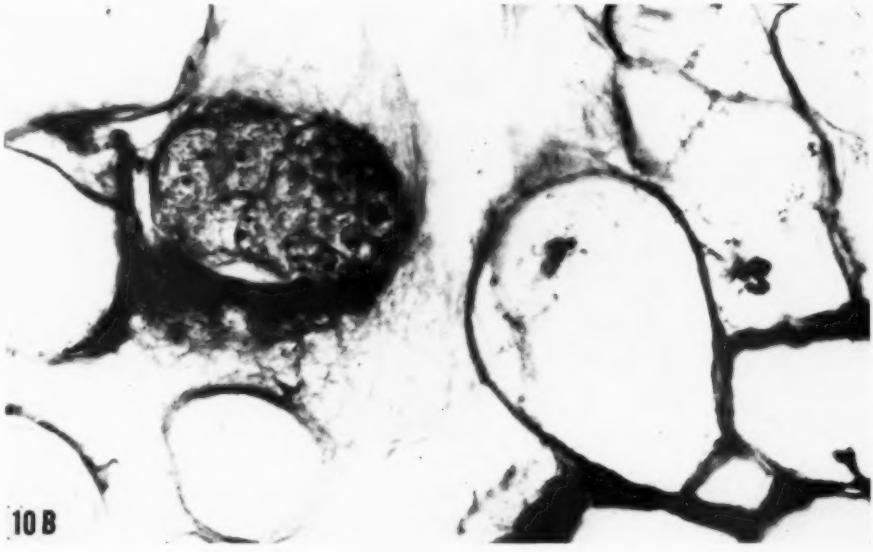
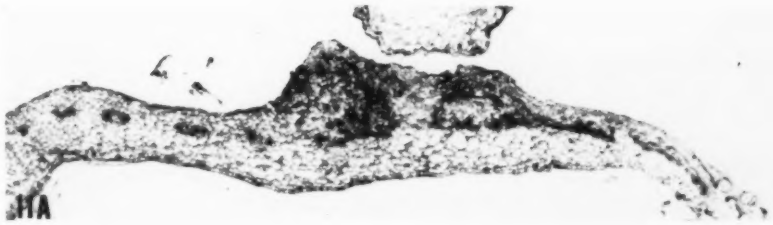

10B

PLATE 153

FIGURE 11.—*A*, Section through a leaflike structure in which the tissues about the two vascular structures seem to have proliferated to form two masses of parenchymatous cells and disorganized vascular elements. × 35. *B*, Part of a tissue shown in *A* under higher magnification. This structure is made up of parenchymatous cells with disoriented lignified vessels. × 560.



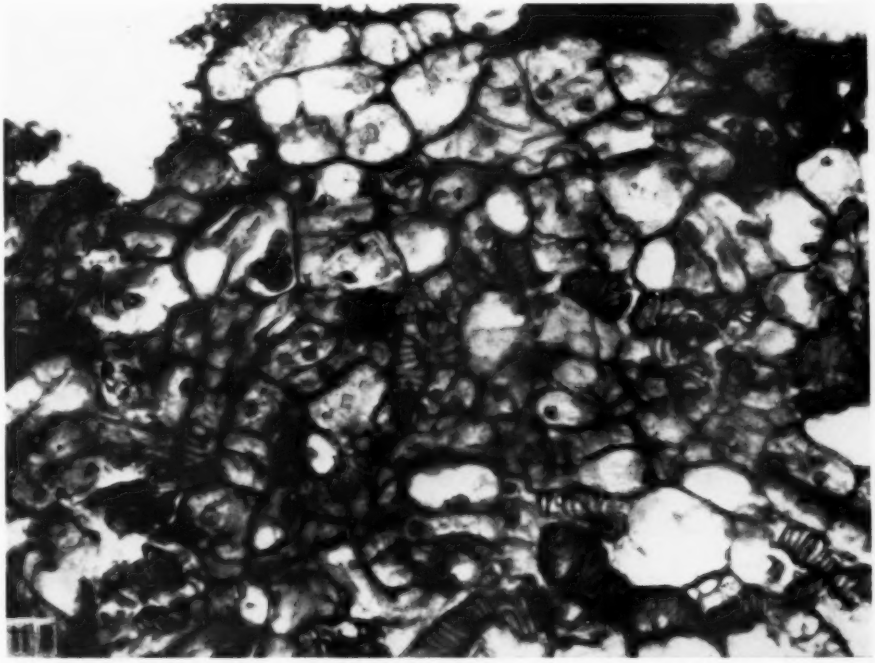
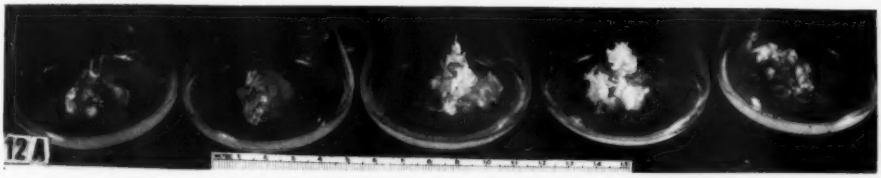


PLATE 154

FIGURE 12.—*A*, A series of clones from culture 7_1x5_4 grown on Wsf, Wsf IAA 5 mg. per liter Wsf D 35 mg. per liter, Wsf B 50 mg. per liter, and Wsf M 50 mg. per liter (read from left to right) showing abortive thallus-like body. × ½. *B*, Section of tissue grown on Wsf plus D, shown in *A*. Giant cells after aberrant division of nucleus. × 560.

FIGURE 13.—A culture (1_24) grown on Wsf with B 50 mg. per liter showing a section of a thallus-like body with nodular proliferation of surface cells. × 100.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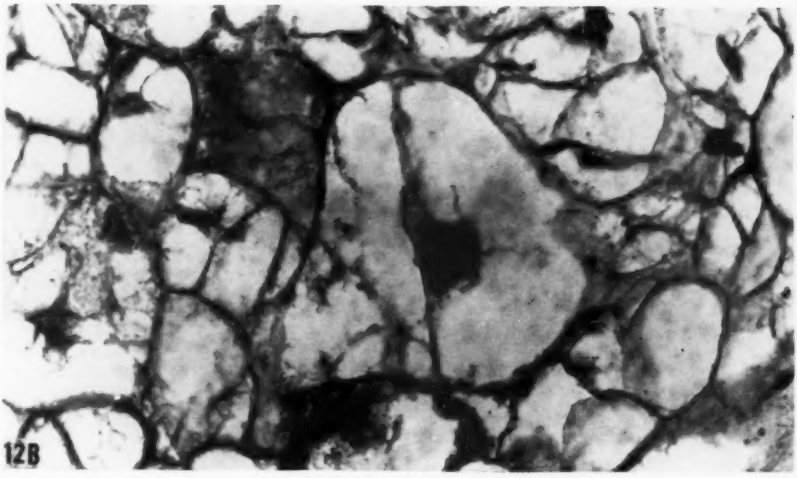

12B

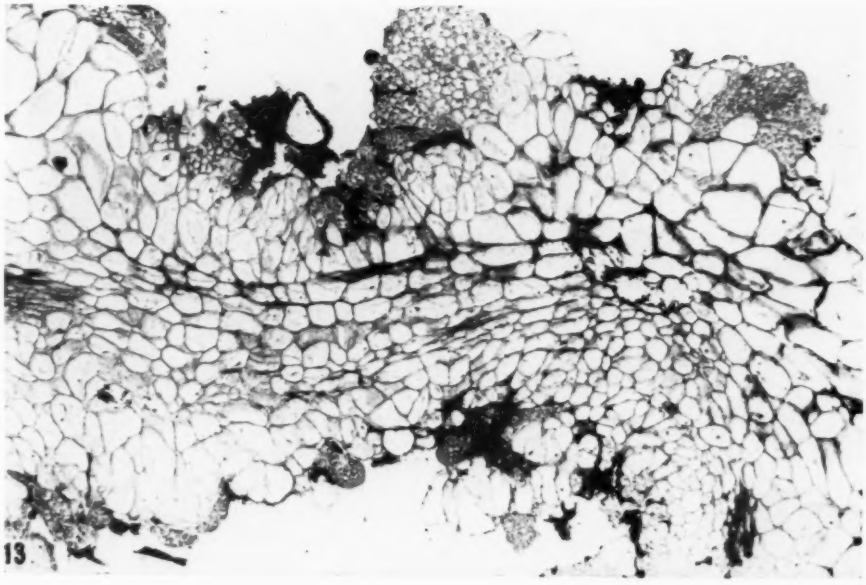
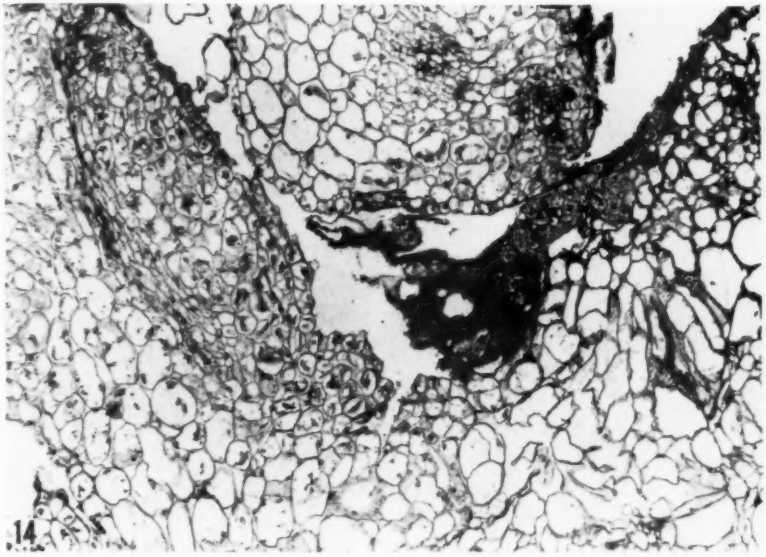

13

PLATE 155

FIGURE 14.—A portion of a thalloid body. Note the organoid that arose from the surface and the proliferation of surface tissue growing against the older parenchymatous tissue. × 100.

FIGURE 15.—Clone 7_1x10_4 grown on *B*. Note new tissue from portion of the surface meristem. × 100.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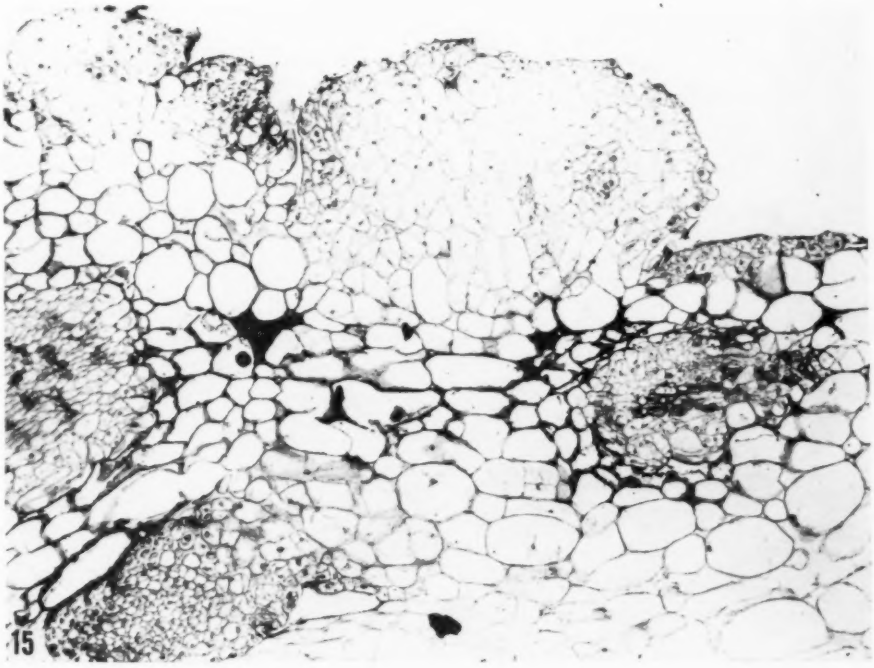
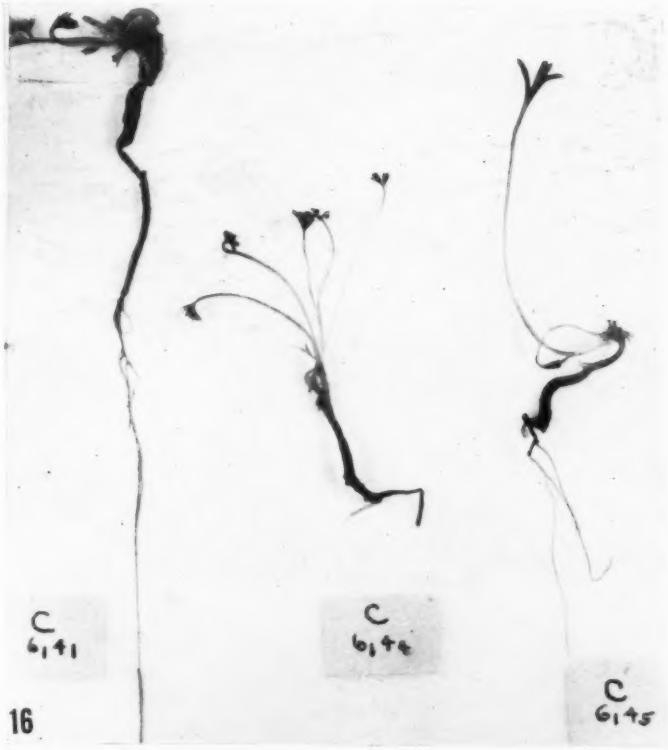

15

PLATE 156

FIGURE 16.—A series of plantlets from B cultures separated from the tissue mass on December 6, 1947, and planted on basic medium, B medium, and M medium (concentration of the carcinogen, 5 mg. per liter), photographed January 2, 1948. × 1½.

FIGURE 17.—Longitudinal section through root of plantlets transferred from medium with B to basic medium. Note crown gall-like appearance of tissue. × 40.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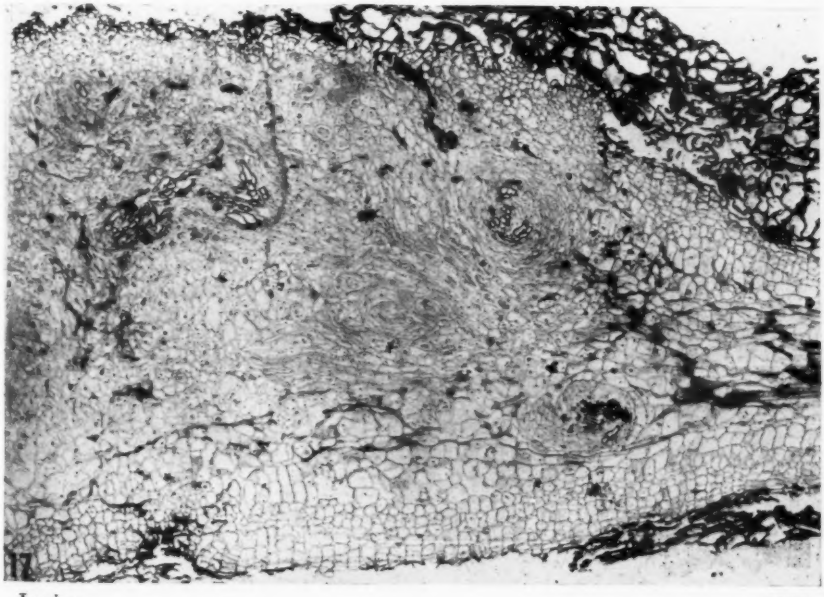
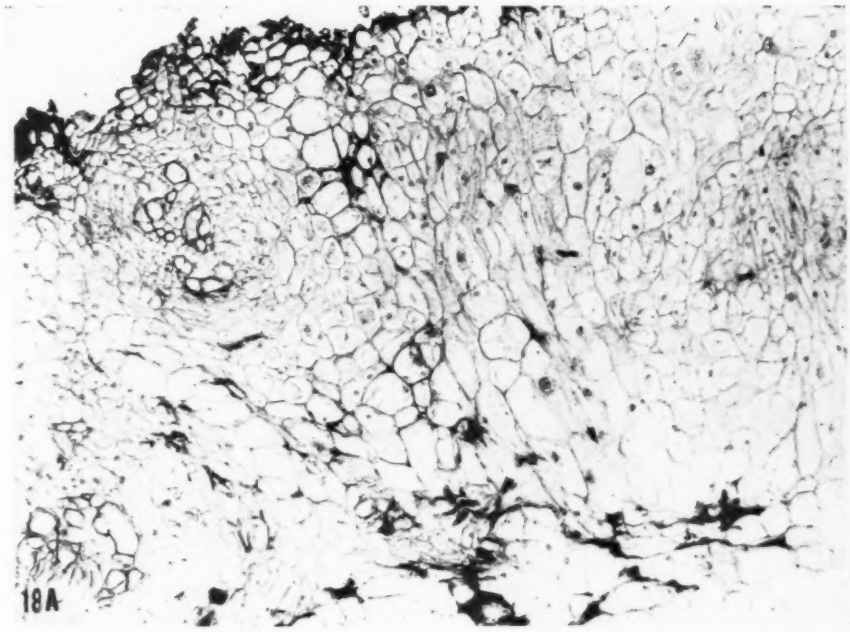

17

PLATE 157

FIGURE 18.—*A*, Portion of figure 17 under higher magnification. × 100. *B*, Stimulated parenchymatous cells from portion of tissue shown in *A*. × 520.


18A

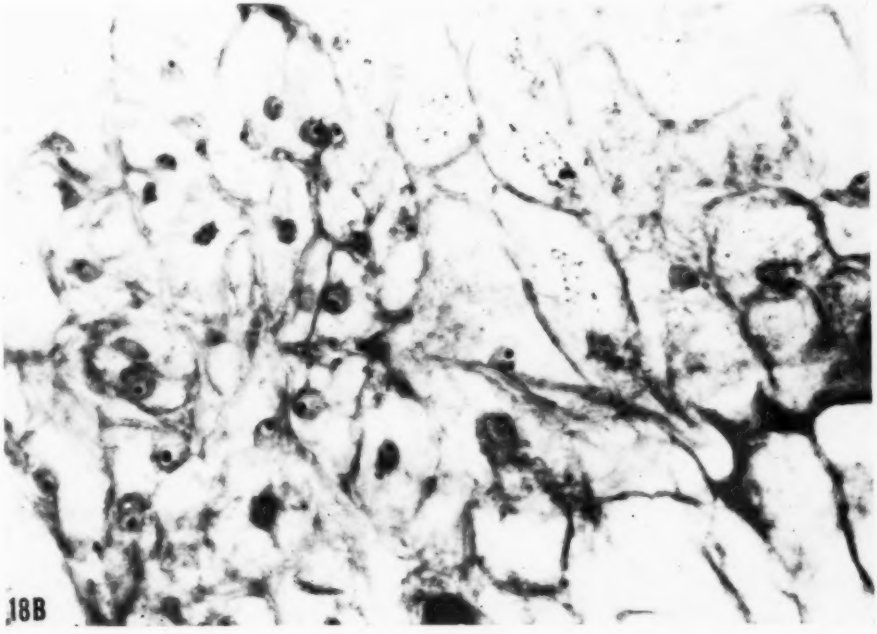
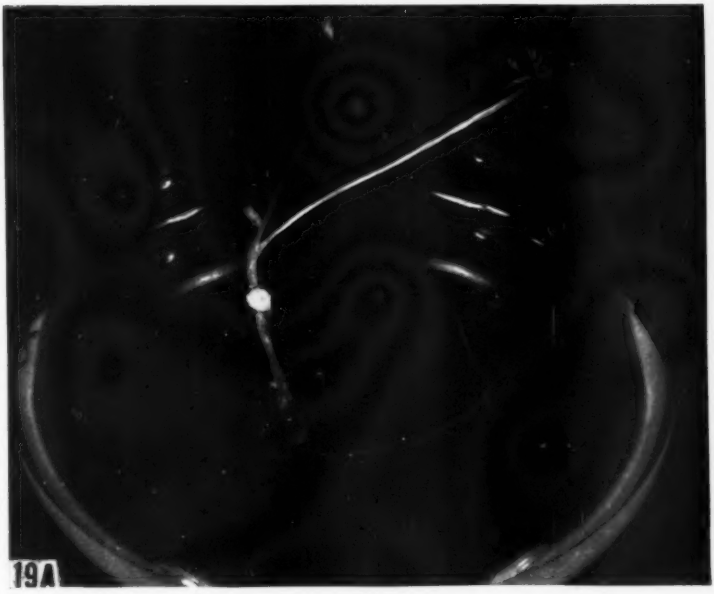
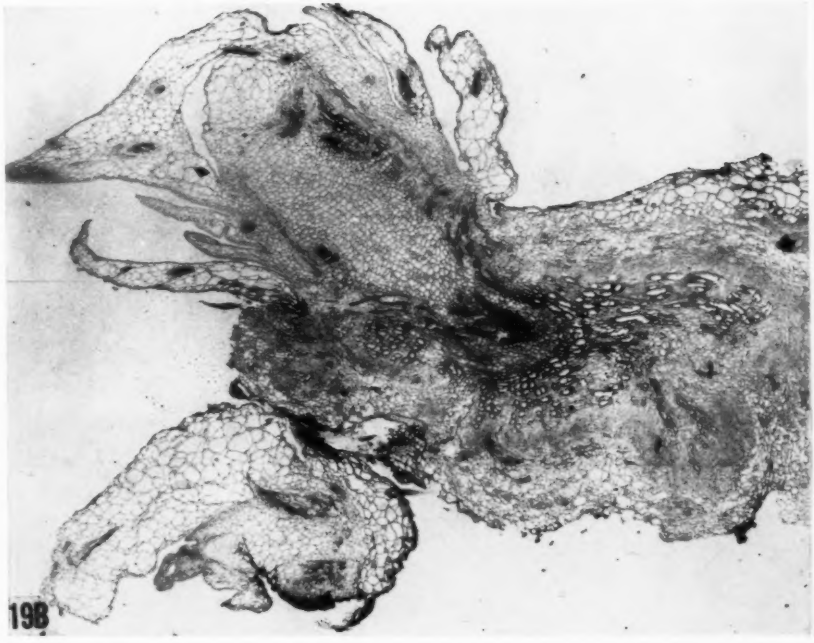

18B

PLATE 158

FIGURE 19.—*A*, Plantlet isolated from tissue mass and tap root below stipule inoculated with a virulent strain of *P. tumefaciens* on January 8, 1948, photographed January 23, 1948. × 2. *B*, Section of a crown gall similar to the one shown in *A*, inoculated January 12, 1948, tissue fixed February 7, 1948. Note crown gall with shoot arising from it. × 25.


19A


19B

HISTOPATHOLOGY OF ESTROGEN-INDUCED TUMORS IN GUINEA PIGS[1]

ELI M. NADEL, M. D.,[2] *National Cancer Institute, National Institutes of Health, Public Health Service, Bethesda, Md., and the Department of Pathology, Washington University School of Medicine, St. Louis, Mo.*

INTRODUCTION

Tumor-like growths have been repeatedly induced in the serosa of the abdominal cavity of guinea pigs subjected to continuous treatment with estrogenic substances (*1*, *2*, *3*). They have been less frequently observed in the thoracic cavity (*4*).

Because of the frequent location of these lesions in the uterus, an analogy has been made to the leiomyoma of women, and the term "fibroid" was early applied to them. This term is still common in the literature, although the precise histologic nature of the nodules has not received adequate attention, and the use of this term is open to question. Other terms employed are fibrosarcoma, fibromyoma, histiocytofibroma, penetrating fibromyoma, and fibroma (*1*, *5*, *6*).

In the experiments reported here the histopathology of these estrogen-induced nodules in the female guinea pig was studied.[3] Special staining techniques have been employed, particularly those which help to differentiate smooth muscle and connective tissue elements. Only animals of an inbred guinea pig strain, Family 13, were used.[4]

MATERIALS AND METHODS

Stilbestrol pellets were implanted subcutaneously in the lateral abdominal wall of each of 20 virgin female guinea pigs. Sixteen of these animals had been oophorectomized,[5] and 4 had been subjected to sham operation

[1] Received for publication October 13, 1949.

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[3] Histopathological and histochemical changes were also observed in the spleen, adrenal glands, and supporting structure of estrogenized guinea pigs. Of especial interest was the observation of splenomegaly, and the presence of large numbers of Foa-Kurloff cells in the spleen of estrogenized guinea pigs. These cells were stained positively by the Schiff periodic-acid method, and they exhibited metachromasia with thionin. A complete report of further investigation of the nature of this peculiar cell is in preparation.

[4] Family 13 is an inbred strain of guinea pigs originally developed by Dr. Sewell Wright, Department of Agriculture. The line has been continued at the National Cancer Institute by Dr. Walter Heston, geneticist.

[5] Oophorectomy was performed under ether anesthesia. Bilateral paravertebral incisions were made, and both ovaries and the adjacent segments of both oviducts were clamped with a hemostat, tied with a single cotton suture, and then excised. The peritoneum was closed by a single cotton suture; the muscle fascia and skin were coapted by a single Michel clip.

within 1 month prior to implantation. Four oophorectomized control animals received implants of cholesterol pellets. The animals were 6 to 9 weeks old at the time of implantation.

Of the 16 oophorectomized animals, 9 received pellet implants containing 20 mg. or more of stilbestrol, while 7 received implants containing less than 20 mg. of stilbestrol. The 4 sham-operated animals received implants containing less than 20 mg. of stilbestrol (table 1). Each of the 4 control animals received implants solely composed of 20 mg. of cholesterol.

All animals were given Purina rabbit chow and wet cabbage daily and water *ad lib.* Carrots were given once weekly. The animals were kept four in a cage.

The stilbestrol pellets were made by the method of Shimkin and White (7), and were prepared from a mixture of equal parts by weight of stilbestrol and cholesterol. The pellets were deposited by trocar subcutaneously between the scapulae.

Eleven of the experimental and all the control animals were killed by cranioconcussion at the conclusion of the experiment. Nine experimental animals were found dead within 8 hours after the last examination (overnight) and autopsies were done immediately.

TABLE 1.—*The distribution of grossly visible tumor nodules in 20 female pigs (Family 13) after implantation of 7½ to 50 mg. stilbestrol for 15 to 96 days*

Animal No.	Duration of treatment	Dose of Stilbestrol	Estrogen effect on vagina and nipples	Oophorectomy	Distribution of nodules							
					Retroperitoneum	Body of uterus	Parietal peritoneum	Stomach	Intestine	Pancreas	Spleen	Gall bladder
	Days	*Mg.*										
1	90	40	+	+	-	-	-	-	-	-	-	-
2	96	50	+	+	+	-	-	-	-	-	-	-
3	35	[1]50	+	+	+	-	-	-	-	-	-	-
4	57	[1]20	+	+	+	+	+	+	+	-	+	-
5	87	[1]20	+	+	+	+	-	+	+	+	+	-
6	90	20	+	+	+	-	-	-	-	-	-	-
7	42	[1]50	+	+	+	+	-	+	-	-	+	-
8	95	50	+	+	+	-	-	-	-	-	-	-
9	96	[1]30	+	+	+	-	-	-	-	-	-	-
10	91	7½	+	+	+	-	-	-	-	-	-	-
11	80	12	-	+	-	-	-	-	-	-	-	-
12	61	7½	+	+	+	-	+	-	+	-	-	-
13	50	8	+	+	+	-	-	-	-	-	-	-
14	50	7½	+	+	+	+	-	-	+	-	-	-
15	55	[1]7½	+	+	+	-	+	-	-	+	-	+
16	15	[1]15	+	+	+	+	-	-	-	-	-	-
17	75	14	-	-	-	-	-	-	-	-	-	-
18	75	15	+	-	-	+	-	-	+	-	-	-
19	75	16	-	-	-	-	-	-	-	-	-	-
20	75	17	-	-	-	-	-	-	-	-	-	-

[1] Pellet recovered at autopsy.

The autopsies were complete except for examination of the central nervous system. Tissues were fixed in Zenker-acetic fluid and in dilute formaldehyde (U. S. P. 1:10). They were embedded in paraffin, sectioned at 6 microns, and stained by the following methods: Hematoxylin and eosin, Mallory's aniline blue, Van Gieson's picric-acid fuchsin, and Mallory's phosphotungstic-acid hematoxylin (PTAH). Silver impregnation for reticulum was done by the Wilder method (*8*).

RESULTS

A summary of the number and location of the grossly visible nodules, the length of exposure to the estrogen pellet, and the dose of estrogen appear in table 1. In general, the response was greater in those animals receiving over 20 mg. of stilbestrol. Of the 20 animals exposed to estrogen, 5 animals (Nos. 1, 11, 17, 19, and 20) failed to develop nodules. Four of these animals, Nos. 11, 17, 19, 20, exhibited no evidence of estrogen effect as indicated by opening of the vaginal orifice (*9*) or enlargement of the nipples (*10*). Two of the 5 animals, Nos. 1 and 11, exhibited cystic dilatation of the uterus. Remains of the estrogen pellets were not recovered from these 5 animals or from 8 (Nos. 2, 6, 8, 10, 12, 13, 14, and 18) of the 15 that developed nodules; presumably they were either completely absorbed or extruded unnoticed. Cholesterol fragments were recovered in 2 of the control animals. No nodules were observed in any of the control animals.

Nodules developed as early as 15 days after exposure to estrogen. These nodules were generally approximately hemispherical, firm, pearly white to grayish white, and ranged in size from 1 to 25 mm. in greatest diameter (figs. 1 and 2). They were located subserosally, in the uterus, spleen, gastrointestinal tract, pancreas, gall bladder, mesentery, and on the under surface of the diaphragm and the parietal peritoneal surface. Nodular lesions appeared regularly in oophorectomized stilbestrol-treated animals at the operative site and around the lower pole of the adjacent kidney. In this position they were firmly embedded in the retroperitoneal tissues but did not extend into the renal parenchyma.

MICROSCOPIC OBSERVATIONS

Histologic Findings

It appeared from histologic examination that the visceral and parietal peritoneum responded to estrogen with a combination of two types of proliferation patterns, one involving the subserosal connective tissue and the other, the mesothelium.

(1) Connective-tissue proliferation was characterized by the formation of flat plaques or more rounded nodules which were visible on the peritoneal surface.

The plaquelike lesions were composed largely of shrunken, hyperchromatic fibroblasts. The nodular lesions varied from those consisting chiefly of older, smaller fibrocytes to those in which young, plump, paler fibroblasts predominated. In both there were endothelial-lined spaces and

clefts occasionally filled with oxyphilic coagulum. Red cells were not identified in these. Lying between the fibroblastic cells was a variable amount of collagen showing differences in intensity of staining.

The nodular lesions could be subdivided roughly into three groups. The first group was relatively acellular, and resembled the flattened plaque-like lesions in that fibroblasts were thin and small, and the collagen was hyalinized and abundant. Nodules of this type developed in the capsule of the spleen (fig. 5) and in the mesentery, and appeared to be an older type of lesion. The second group was more cellular, the cells were larger, and in addition to the fibroblasts, a number of smooth muscle cells were present. These smooth muscle cells were confined to the periphery of the nodule (fig. 7), and may well have been separated from the parent tissue in the development of the nodule. This type of nodule developed in the intestine (fig. 6) and in the uterus (fig. 3), and appeared to be a younger lesion. In the third group, the nodules extended from the serosal surface into the underlying tissues. Nodules of this type were the largest of the three varieties. They exhibited the greatest cellularity (fig. 9), and not infrequently contained mitotic figures. No smooth muscle elements were seen in this type of nodule. Such nodules were found in the retroperitoneal area around the lower pole of the kidney and extended into the skeletal muscle of the trunk. They also developed in the parietal peritoneum under the diaphragm (fig. 8), in the mesometrium, and in the pancreas (fig. 4). Infiltration of skeletal muscle by the proliferating fibroblasts resulted in compression atrophy of the muscle fibers. Multinucleated sarcolemmal giant cells and remnants of atrophic muscle cells were seen as islands in the mass of actively-growing fibroblasts (fig. 10). Penetration of the pancreas by the actively-growing fibroblastic tissue resulted in atrophy and destruction of parenchymatous cells. Surviving remnants of isolated pancreatic tissue were seen deep in the mass of invading cells.

The experimentally-produced nodules therefore varied from a relatively acellular fibroma type to an infiltrating fibroblastic tumor, similar to the variation seen in the "desmoid" tumor in man (fig. 11) [Compare with fig. 10.]

(2) The type of response in which the mesothelial cells actively participated consisted of a proliferation, budding, and exfoliation of peritoneal serosal lining cells (fig. 17). In some instances this resulted in a papillary structure composed of a central core of connective tissue covered by a single layer of cells derived from the serosa (fig. 18). In others, infolding, invagination and downgrowth of the mesothelium resulted in the formation of clefts or crypts lined by mesothelial cells (fig. 12). With further downgrowth, a pinching off of the original connection to the peritoneal cavity took place, leaving the mesothelial cells trapped in the deeper tissues where they formed small endothelial-lined cavities. The mesothelial participation was most frequently present in nodules of the penetrating type, but it was also seen to a lesser degree in the other nodules and even as a surface reaction in areas without tumors. The down-

growth of mesothelium and the presence of trapped mesothelial cells suggested that many of the more deeply-situated fibroblastic cells in the tumor nodules might arise from such elongated, invaginated, heterotopic mesothelial cells.

Cytologic Findings

In studying the cytologic detail of these tumors induced by estrogen in the guinea pig, comparison of the cells in the tumor with the normally-located smooth muscle cells in the same section of uterus or intestine was a helpful control measure.

Study of sections of the nodules stained by the Van Gieson and Mallory aniline-blue method showed the presence of small-to-large amounts of collagen and medium-to-large, plump, spindle or stellate cells in interlacing bundles. Nuclei were large and oval, surrounded by a thin basophilic nuclear rim and enclosing pale, dustlike granules and one or two small, lightly chromatic nucleoli. The cytoplasm was indistinct in areas, the cell borders appearing to interdigitate loosely. Where the collagen was abundant, the cells were fewer in number and the nuclei were smaller, and often hyperchromatic and pyknotic. The cytoplasm of the larger cells often took an off-shade of yellow green with the Van Gieson stain. With the Mallory aniline-blue stain, the cells took a pale reddish coloration. In the cytoplasm and along the borders of the cells fibroglial elements stained deep red (fig. 14). These elements were also present in the intercellular connective tissue near the cytoplasmic boundaries. In the normal smooth muscle areas of the intestine or uterus, the smooth muscle cells were stained a bright yellow with Van Gieson in contrast to the yellow green of the large fibroblastic cells of the tumor nodule. In addition, intercellular glial elements could not be seen in the smooth muscle areas with the Mallory aniline-blue stain. With the phosphotungstic-acid hematoxylin method, delicate blue fibroglial fibrils (fig. 15) could be seen in the cytoplasm of the cells of the tumor nodules. These glial elements could be seen to sweep from cell to cell or fan out from the cytoplasmic peripheral border. In the same section, smooth muscle cells of the muscle coats were seen to be associated with blunt, thick, heavily blue-stained myoglial fibrils along the border of the cytoplasmic body. In addition individual "hockey-stick" shaped myofibrils could be demonstrated (fig. 13). In other areas contraction nodes could be seen in bundles of smooth muscle cells.

With silver impregnation reticulum surrounded each tumor cell individually (fig. 16). In contrast, reticulum surrounded groups of smooth muscle cells in addition to individual smooth muscle cells in the nontumor bearing areas of the uterus and intestine.

With the aid of special stains, the tumor nodules appeared to be composed of fibroblasts of varying ages. There was no problem even with hematoxylin and eosin stains in deciding upon the nature of the smaller cells in the older type of lesion. The problem presented itself where the nodules contained larger younger fibroblasts. Were only the Van Gieson stain relied upon, the type of cell present in the lesion could not have been

determined with certainty because of its atypical staining qualities. It is well known that "young connective tissue may stain yellow rather than red with Van Gieson's stain—and that it may be difficult to distinguish by this method fibromas from leiomyomas" (*11*). The fibroglia which was present in large amounts in the tumor could be demonstrated by either of Mallory's two methods, though the PTAH method in our hands seemed the better of the two. By the PTAH method, it was possible to distinguish the delicate tonofibril (*12*) or fibroglial (*13*) elements from the coarser myoglial elements, and also to demonstrate the smooth muscle contractile myofibrils (figs. 13 and 15).

It is known that the tissues lining the peritoneal cavity are derived from embryonic mesenchyme. The concept that pluripotential mesenchyme-like cells exist in the adult has many supporters (*14*, *15*), and it has been helpful in explaining the apparent intermutability of the cells of the connective-tissue series. If we consider that the proliferating estrogen-induced tumors contain incompletely differentiated mesenchymal cells, it is possible to understand why these cells may have been considered smooth muscle cells by other observers. Conceivably these mesenchymal cells could be misinterpreted as smooth muscle cells if the mesenchymal cells were stained solely by the Van Gieson method at the stage when they had not yet formed collagen or if the delicate, elusive, fibroglial elements were not demonstrated or had not yet developed.

DISCUSSION

The infiltrating fibroblastic nodule which develops after estrogen stimulation in guinea pigs is strikingly similar to the desmoid tumor in man first described by MacFarlane in 1832 [6] and so named by Mueller in 1838.[7] Pack and Ehrlich state that "the microscopic appearance of the desmoid tumor is that of a fibroma plus the presence of striated muscle fibers in various stages of atrophy. The histological picture may vary from that of an acellular fibroma to that of an acellular low-grade fibrosarcoma. The desmoid tumor differs from the ordinary fibroma in that it is often unencapsulated and possesses infiltrative tendencies. Characteristically it always compresses, invades, and destroys muscle. The usual encapsulated fibroma is never aggressive. Occasionally desmoids undergo transformation into low-grade fibrosarcomas, but they never involve the regional lymph nodes or produce distant metastases" (*16*). This picture is not dissimilar to the lesions described above in the experimentally induced tumor. Musgrove and McDonald (*17*) histologically compared fibrosarcomas which were clinically of low-grade malignancy with extra-abdominal desmoids. They reported that fibrosarcomas were more circumscribed, more cellular, had more mitotic figures than did desmoids, and, in addition, that fibrosarcomas contained tumor giant cells and exhibited greater cellular pleomorphism. However, in a recent

[6] MacFarlane, J.: Clinical reports of the surgical practice of the Glasgow Royal Infirmary, as quoted by Pack and Ehrlich (*16*) and Musgrove and McDonald (*17*).

[7] Mueller, J.: As quoted by Pack and Ehrlich (*16*) and Musgrove and McDonald (*17*).

review, Stout (*18*) placed desmoid tumors in that group of fibrous growths "which may or may not be neoplastic," and stated that he did not believe it was possible to distinguish a true neoplasm, such as a fibrosarcoma, from what he called a questionable neoplasm, such as a desmoid, purely on the basis of histopathology. Since metastases did not occur from experimental tumors herein described, it is difficult to classify them as fibrosarcoma. In addition, the experimental tumor is known to regress when the estrogenic treatment is suspended, indicating that it is a dependent growth (*6*, *19*). However, its infiltrative qualities differentiate it from the ordinary concept of a fibroma. Because of the close morphologic resemblance between some of the variants of the experimental tumor and the so-called "desmoid tumor in man," the author feels justified in labeling such experimental tumors in the guinea pig as the "desmoid" type, albeit there are ambiguities inherent in the term.

In addition to the connective tissue, the mesothelium responded to estrogen, with proliferation of the peritoneal mesothelial cells, and pseudoacinar development. These forms are simliar to the clumps of peritoneal-lining cells noted in ascitic-fluid sediments, which may be especially prominent in patients with hepatic cirrhosis. In some instances (*20*) the presence of such acinar formations may well lead to an erroneous diagnosis of neoplasia (figs. 19 and 20). Foot states (*21*) that "in hepatic cirrhosis there is usually a massive proliferation and desquamation of peritoneal cells," and that though there are many mitotic figures, these cells are not tumor cells, despite their "quasi-neoplastic" appearance. He suggests that ascitic fluid is a particularly good culture medium for the growth of such cells. An attractive though tenuous hypothesis is the analogy between the mesothelial proliferation in estrogen-treated guinea pigs and in the state of hyperestrogenism known to occur secondary to liver disease (*22*).

Previous investigators have commented upon the activity of the peritoneal-lining cells in estrogen-treated guinea pigs (*5*), (*6*), (*19*), and (*23*). Lipschütz considered the possibility that these nodules might be derived from mesothelial cells. Woodruff described crypt formation. The significance of this proliferation has not been stressed, however; and there has been tacit acceptance of the belief that these tumor nodules arise from multiple independent foci (*4*, *19*). The presence of exfoliated mesothelial-cell proliferations in the animals studied in this series strongly suggests that a seeding of the peritoneum should also be considered as a mode of origin of those tumors.

SUMMARY

The cytologic and histologic nature of the estrogen-induced tumor in the Family 13 guinea pig was studied with the aid of special staining methods. The demonstration of fibroglia in the tumor and the absence of myoglia in all but a few peripheral foci of some of the tumors indicate the essential fibroblastic nature of the tumor. The few smooth muscle cells found in some of the tumor nodules were thought to be components

of the tissues in which the nodule developed and not an integral part of the growth.

The experimentally induced nodules varied in structure from that of a relatively acellular fibroma to that of a locally invasive, infiltrative type of fibroblastic tumor involving skeletal muscle. Since metastases and cellular and nuclear pleomorphism were absent, the tumor is not a true highly malignant fibrosarcoma. Because of its invasiveness, it is not the usual type of fibroma. In the absence of smooth muscle as an integral part of the tumor, it is not a leiomyoma. The tumor nodules most closely resemble those tumors known in man as "desmoid tumors" and should be classified in that group.

Less prominent than the growth of nodules was proliferation of the mesothelium. Such growth gave rise to pseudo-acinar forms similar to those in human ascitic fluid and especially prominent in patients with hepatic cirrhosis.

The misleading term "fibroid" used in the sense of a leiomyoma or fibromyoma should be discarded as a descriptive term for these estrogen-induced tumors; the term "desmoid" type has been suggested in its place.

REFERENCES

(1) Nelson, W. O.: Endometrial and myometrial changes, including fibromyomatous nodules, induced in uterus of guinea pig by prolonged administration of oestrogenic hormone. Anat. Rec. 68: 99–102 (1937).

(2) Lipschütz, A., and Iglesias, R.: Multiples tumeurs uterines et extragenitales provoquees par le benzoate d'oestradiol. Compt. rend. Soc. de biol. 129: 519–524 (1938).

(3) Moricard, R., and Cauchoix, J.: Realisation de volumineux fibromes chez la femelle de cobaye par l'injection de benzoate de dihydrofolliculine. Compt. rend. Soc. de biol. 129: 556–558 (1938).

(4) Bruzzone, S., Elgueta, H., Iglesias, R., and Lipschütz, A.: Oestrogen-induced fibroids of the thoracic serosa. Brit. J. Cancer 2: 267–273 (1948).

(5) Mosinger, M.: La fibromyomatose et fibrosarcomatose multiple experimentale d'origine oestrogene chez le cobaye. Compt. rend. Soc. de biol. 140: 323–324 (1946).

(6) Lipschütz, A.: Induction and prevention of abdominal fibroids by steroid hormones, and their bearing on growth and development. Cold Spring Harbor Symp., Quant. Biol. 10: 79–90 (1942).

(7) Shimkin, M. B., and White, J.: Absorption rate of hormone-cholesterol pellets. Endocrinology 29: 1020–1025 (1941).

(8) Mallory, F. B., and Wright, J. H.: Pathological Technique. W. B. Saunders Co., Philadelphia (1908).

(9) Hartman, C. G., Littrell, J. L., and Tom, J.: Weanling guinea pig as test object for estrogen assays. Endocrinology 39: 120–130 (1946).

(10) Gomez, E. T., and Turner, C. W.: Noneffect of estrogenic hormones on mammary gland of hypophysectomized guinea pig. Proc. Soc. Exper. Biol. & Med. 34: 320–322 (1936).

(11) Atlas of Dermal Pathology. Amer. Reg. of Path. p. 84. Registry Press, Washington, D. C. (1948).

(12) Maximow: Beitz. Z. path. Anat. U. Z. allg. path., Supp. 5: No. 1, 1902, quoted in Handbook of Hematology, Downey, Hal, Editor, II: Section XXVII, 1342–1344, Hoeber (1937).

(13) Mallory, F. B.: A hitherto undescribed fibrillar substance produced by connective tissue cells. J. Med. Research 10: 334–341 (1903–1904).

(*14*) PATTEN, B. M.: Human Embryology. Blakiston Co., Philadelphia (1947).
(*15*) WILLIS, R. A.: Pathology of Tumors. C. V. Mosby Co., St. Louis, Mo. (1948).
(*16*) PACK, G. T., and EHRLICH, H. E.: Neoplasms of anterior abdominal wall with special consideration of desmoid tumors; experience with 391 cases and collective review of literature. Internat. Abstr. Surg. 79: 177–198 (1944).
(*17*) MUSGROVE, J. E., and MCDONALD, J. R.: Extra-abdominal desmoid tumors; their differential diagnosis and treatment. Arch. Path. 45: 513–540 (1948).
(*18*) STOUT, A. P.: Fibrosarcoma; malignant tumor of fibroblasts. Cancer 1: 30–63 (1948).
(*19*) LIPSCHÜTZ, A., and VARGAS, L., Jr.: Structure and origin of uterine and extragenital fibroids induced experimentally in guinea pig by prolonged administration of estrogens. Cancer Research 1: 236–249 (1941).
(*20*) SCHLESINGER, M. J.: Carcinoma cells in thoracic and in abdominal fluids. Arch. Path. 28: 283–297 (1939).
(*21*) FOOT, N. C.: Special methods for the diagnosis of tumors in the laboratory. Am. J. M. Sc. 215: No. 5, 479–486 (1948).
(*22*) GLASS, S. J., EDMONSON, H. A., and SOLL, S. N.: Excretion of estrogen after injection of estradiol and estrone into men with cirrhosis of liver. J. Clin. Endocrinol. 4: 54–57 (1944).
(*23*) WOODRUFF, L. M.: Tumors produced by estradiol benzoate in guinea pig. Cancer Research 1: 367–370 (1941).

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PLATE 159

FIGURE 1.—At post-mortem examination nodules were grossly visible on the greater curvature of the stomach, the small intestine, spleen, the parietal peritoneum (under rib) and uterus.

FIGURE 2.—Uterus of above showing large apical tumors and pararenal cuffs, and numerous nodules on the two uterine horns.

1

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PLATE 160

FIGURE 3.—Cellular type of tumor in the uterus. The smooth muscle of the uterus and the elements of the tumor are easily distinguishable. Phosphotungstic-acid hematoxylin. × 12.

FIGURE 4.—Tumor extending into and replacing pancreatic tissue. Hematoxylin and eosin. × 12.

FIGURE 5.—Relatively acellular tumor on the surface of the spleen. Nuclei are small and hyperchromatic; stroma is abundant and hyalinized. The tumor is extracapsular and compresses the splenic tissue. Slight mesothelial proliferation. Hematoxylin and eosin. × 80.

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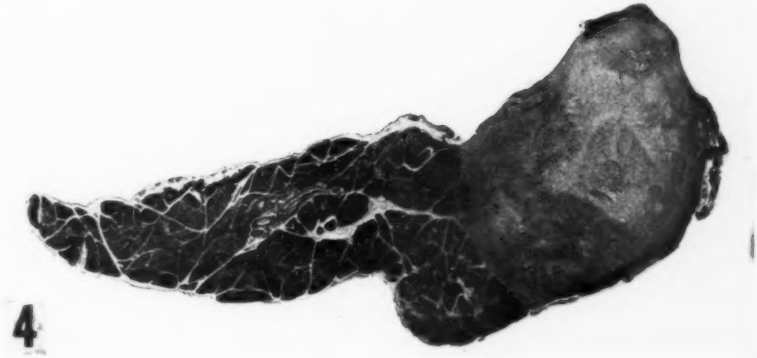

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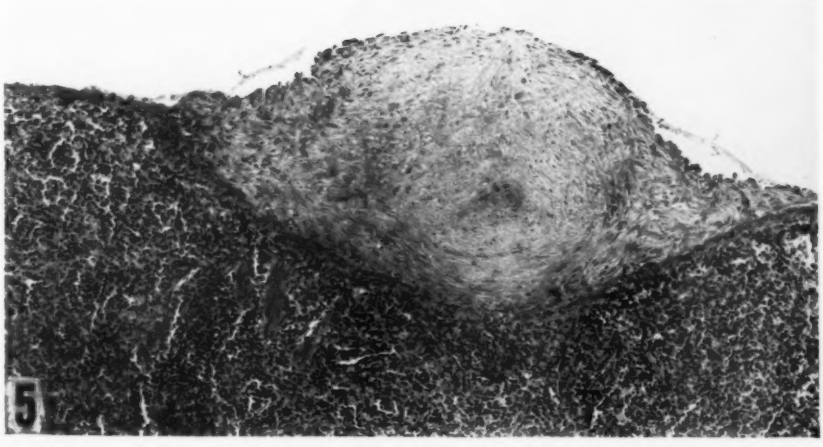

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PLATE 161

FIGURE 6.—Very cellular tumor in the wall of the intestine. Compression of the muscle is less pronounced. Hematoxylin and eosin. × 12.

FIGURE 7.—Junction of smooth muscle of the intestine and the tumor. Demarcation not distinct. Hematoxylin and eosin. × 65.

6

7

PLATE 162

FIGURE 8.—Penetration of tumor into the diaphragm from the parietal peritoneum. Lowest three ribs are shown in cross-section. Hematoxylin and eosin. × 6.

FIGURE 9.—Central portion of the tumor showing interlacing bundles of fibroblastic cells with a fairly abundant collagenous stroma. Endothelial-lined spaces are also seen. Hematoxylin and eosin. × 120.

FIGURE 10.—Extension of tumor into skeletal muscle. Multinucleated muscle cells are seen surrounded by tumor tissue. Hematoxylin and eosin. × 126.

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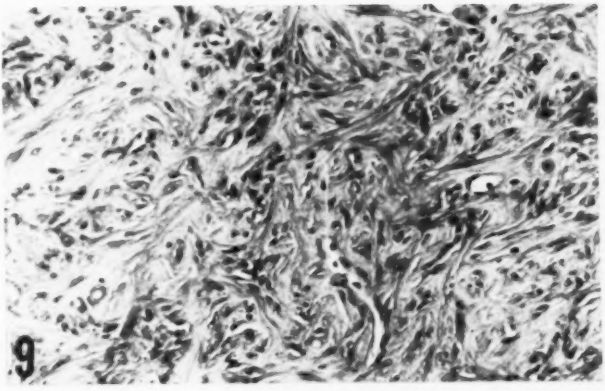
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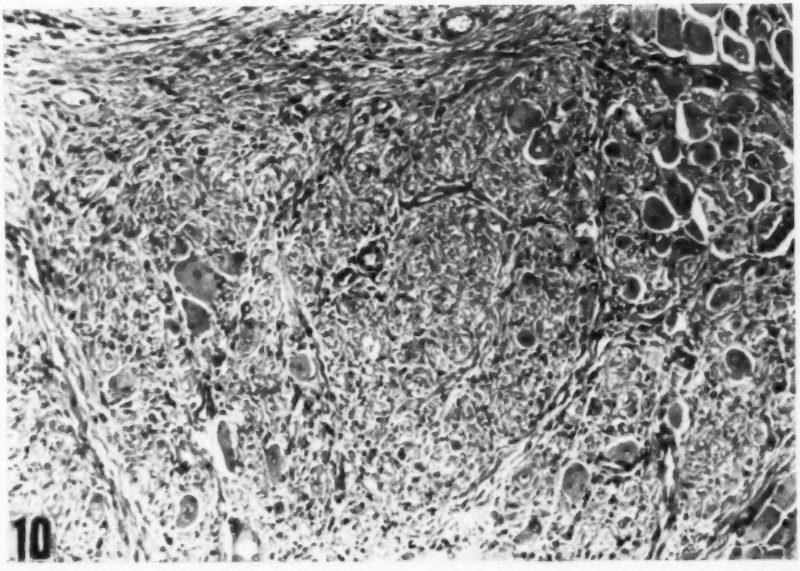
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PLATE 163

FIGURE 11.—Desmoid tumor in man showing extension into skeletal muscle. Hematoxylin and eosin. × 140.*

FIGURE 12.—Mesothelial projection and crypts. Hematoxylin and eosin. × 220.

*Photographed by the Department of Surgical Pathology, Barnes Hospital, St. Louis, Mo.

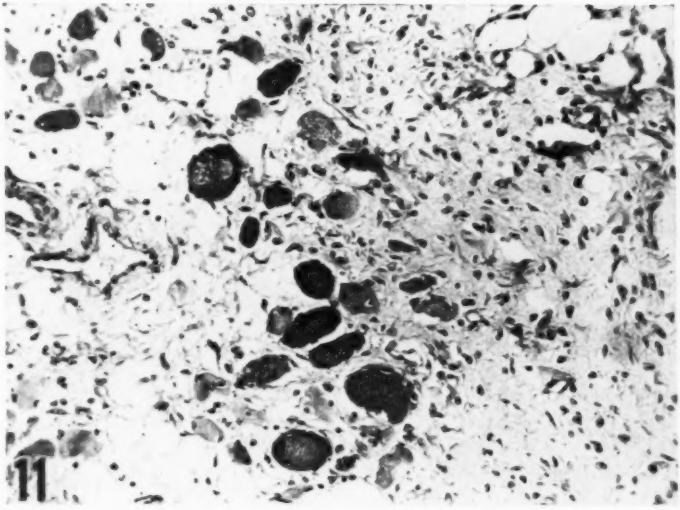
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PLATE 164

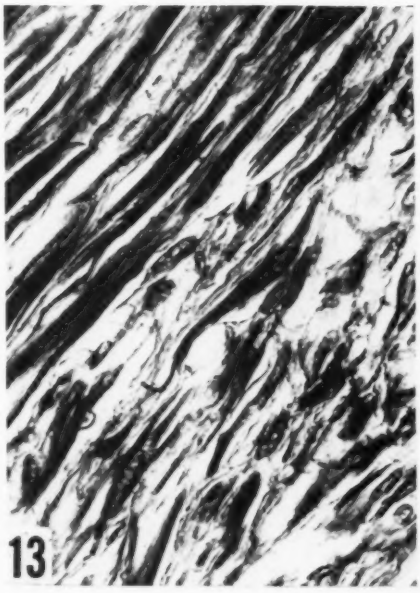
FIGURE 13.*—Individual smooth muscle cells of the uterine wall from the same section as in figure 15. Smooth muscle cells are slightly spread apart because of the action of estrogen. In the central cell note two thick coarse cytoplasmic myoglial fibers, one of which is continuous with the thinner hockey-stick shaped contractile element known as the myofibril. Phosphotungstic-acid hematoxylin. × 600.

FIGURE 14.—Fibroblastic tumor. Fibroglia stains red and can be seen as thin fibrillae in the cytoplasm and intercellular connective tissue. Mallory's aniline blue method. × 260.

FIGURE 15.—Fibroblastic tumor. Fibroglia stains blue and consists of delicate strands in the cytoplasm of the large plump fibroblasts. The glial fibers fan out from the periphery of the cell body and run in roughly parallel paths in the intercellular connective tissue. Phosphotungstic-acid hematoxylin. × 400.

FIGURE 16.—Section through fibroblastic tumor in uterus. Reticulum surrounds individual fibroblastic cells. Silver impregnation, Wilder method. × 160.

*Figs. 13-17 were photographed by the Illustration Department, Washington University School of Medicine, St. Louis, Mo.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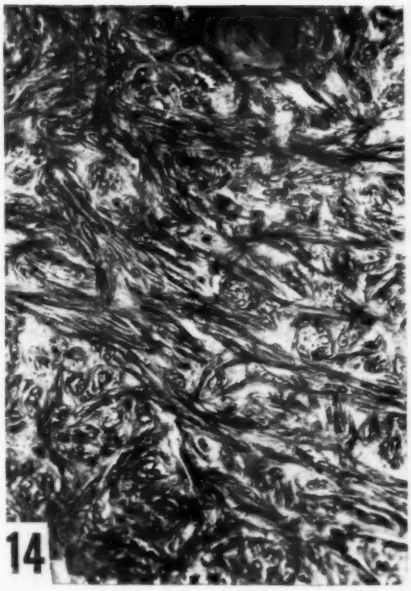
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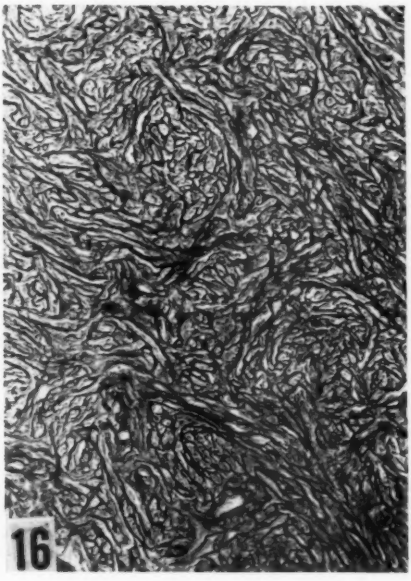
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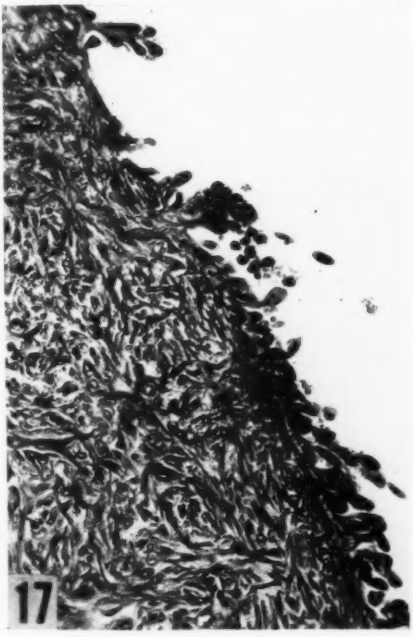
PLATE 165

FIGURE 17.—Mesothelial proliferation over a tumor nodule with budding and exfoliation of peritoneal lining cells. Phosphotungstic-acid hematoxylin. × 250.

FIGURE 18.—Cleft in the tumor lined by cells of the serosal mesothelium. Hematoxylin and eosin. × 220.

FIGURE 19* and 20.**—Pseudo-acinar formations in peritoneal fluid of patients with ascites due to cirrhosis of liver. Hematoxylin and eosin. × 150 and × 100 respectively.

*From the Department of Surgical Pathology, Barnes Hospital, St. Louis, Mo.
**From the Department of Pathology, Jewish Hospital, St. Louis, Mo.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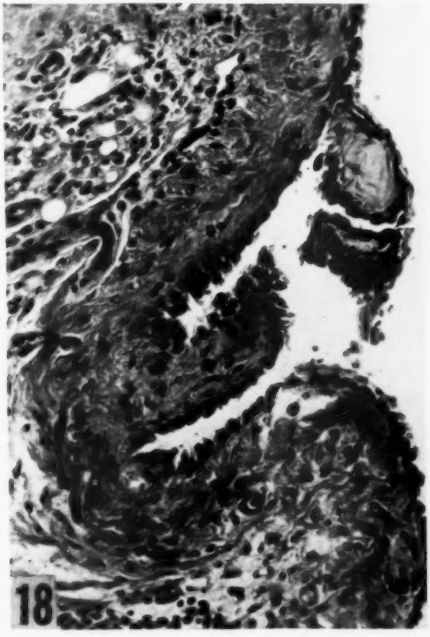
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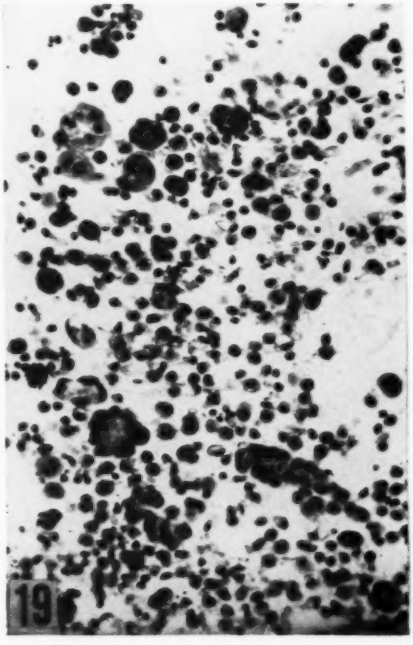
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PRODUCTION OF MALIGNANCY *IN VITRO*. X. CONTINUED DESCRIPTION OF CELLS AT THE GLASS INTERFACE OF THE CULTURES [1]

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INTRODUCTION

In an earlier article (*1*) experiments were described in which a parent strain of fibroblasts [3] from the subcutaneous fibrous and adipose connective tissue of a young, adult, male, C_3H mouse was first transplanted *in vitro* October 18, 1940, and was grown there in an entirely heterologous medium. Cell strains taken from this parent strain were treated with 20-methylcholanthrene in a concentration of approximately 1 microgram of carcinogen per milliliter of fluid culture medium for intervals ranging from 6 to 406 days. Most of this study was carried out on 6 cell strains. Five of these, designated as strains H, J, L, N, and O, were treated with carcinogen for 6, 32, 111, 184, and 406 days respectively. The sixth, designated strain D, was never treated with carcinogen but was carried as an untreated control strain. Morphologic alterations were observed in the cells of each of the treated strains, and the extent of these changes for each strain was directly correlated with the duration of its carcinogen treatment. The nature of the changes was such that the fibroblasts at the glass interface [4] of the culture shortened and became increasingly coherent to the cells laterally adjacent.

About 120 days after the last of these treated cultures showed such alteration, the cells of the untreated control cultures began to show a change similar in morphology but more limited in extent. The cause of this change in the untreated controls was not indicated by the data obtained.

When studied at a later period, cell changes at the fluid interface of the cultures (*2*) of these 6 strains were quite different in appearance from the

[1] Received for publication November 30, 1949.

[2] Acknowledgment is also made of the technical assistance of Mary F. Brown, Thomas H. Stark, Nancy P. Straus, Josephine Kahler, Emily Duchesne, Rosslyn Wallace, Grady Carter, Leslie Hall, and Ellison Peppers.

[3] In this paper the original cell strain will be designated the *parent strain*, each major subordinate group of cultures that arose from it will be designated as a *strain*. Subordinate groups of cultures in each strain will be designated as culture *lines*.

[4] The terms *glass interface* and *fluid interface* of the culture are used to indicate the position of cells growing in the plasma adjacent to the glass surface of the culture flask and adjacent to the supernatant fluid culture medium respectively.

changes at the glass interface. Particularly in strains treated for longer intervals, the culture morphology was no less characteristic.

The description of the growth of the cultures of these 6 cell strains and a comparison of their culture design at the glass interface was carried up to May 15, 1943. It is the purpose of the present paper:

1. To correlate more clearly, by means of a chart, the relationship of cell strains and cell lines used in the studies, and the chronological relationships of the various studies in this series.

2. To continue the account of the general growth behavior of these 6 culture strains subsequent to May 15, 1943.

3. To continue a description of the cells at the glass interface of these strains subsequent to May 15, 1943, with particular reference to the nature and permanence of the observed cell changes.

MATERIALS, METHODS, AND CORRELATION OF STUDIES

The methods used in the planting, maintenance, and photography of the cultures have already been described (*1, 2, 3, 4*). While some of the descriptions of cell design at the glass interface have been taken from written culture records, the majority of the data have been compiled from a detailed comparison of photomicrographic records of the cell strains described. The number of photographic negatives used in this comparison exceeded 1,100, nearly all of which were 8- by 10-inch size. All photomicrographs presented in this paper are areas of contact prints from such representative 8- by 10-inch negatives of cells at the glass interface of the cultures. The magnification is uniformly ×200.

To reduce the hazard of loss of any culture strains from bacterial contamination, each strain was generally carried in at least 2 culture groups, each of which received culture media from a different lot. Carrying such duplicate groups of cultures unintentionally resulted in segregation and preservation of several rather definite lines of culture inheritance in each of the strains. While no attempt can be made to detail the ancestry of all cultures in this study, the more important strains and lines are indicated in text-figure 1.

All photographic records of each strain were correlated with the genealogical records of that strain. While many of the cell lines were closed so early that no effective comparison could be made between their cultures, of those which could be compared, the only cell strain showing any substantial difference between its cell lines was the control strain D. This variation will be described later.

A tabulation of the approximate number of cultures carried in the whole course of this study from the time the 6 cell strains were originated from the parent strain up until February 1948, when 5 of the strains were closed, is as follows:

Strain D in excess of 2,000 cultures.
Strain H in excess of 1,700 cultures.
Strain J in excess of 1,600 cultures.
Strain L in excess of 2,200 cultures.

Strain N in excess of 1,300 cultures.
Strain O in excess of 2,200 cultures.
Total number of cultures in excess of 11,000.

Many of these cultures were never used for detailed comparative cell study, but were used for animal injection or other types of work. Even with cultures used for injection and other purposes, however, microscopic examination routinely made was such that any substantial deviation from the cell forms described for each of the cell strains would have been recognized.

RESULTS

Strain D control cultures.—Subsequent to May 15, 1943, (*1*) growth of strain D control cultures continued luxuriant.

Within the period from December 1942 to March 1943, the cells at the glass interface of this strain were in general radially oriented relative to the explant and showed a degree of shortening and of lateral cohesion barely recognizably altered from the normal. Comparison of photographs of cultures of this strain in January 1944 showed a slightly greater variability in cell design in the cultures of the β line of this strain than in the α. In a few cases the lateral cohesion and shortening of the β line cells even equaled that seen in strain H cultures. Considered as a whole, however, the cells of this D strain showed no appreciable change in their morphology from March 1943 up to the time the cultures were closed in February 1948. This uniformity of morphology is well illustrated by comparing figures 1 and 2 with earlier ones published (*1*).

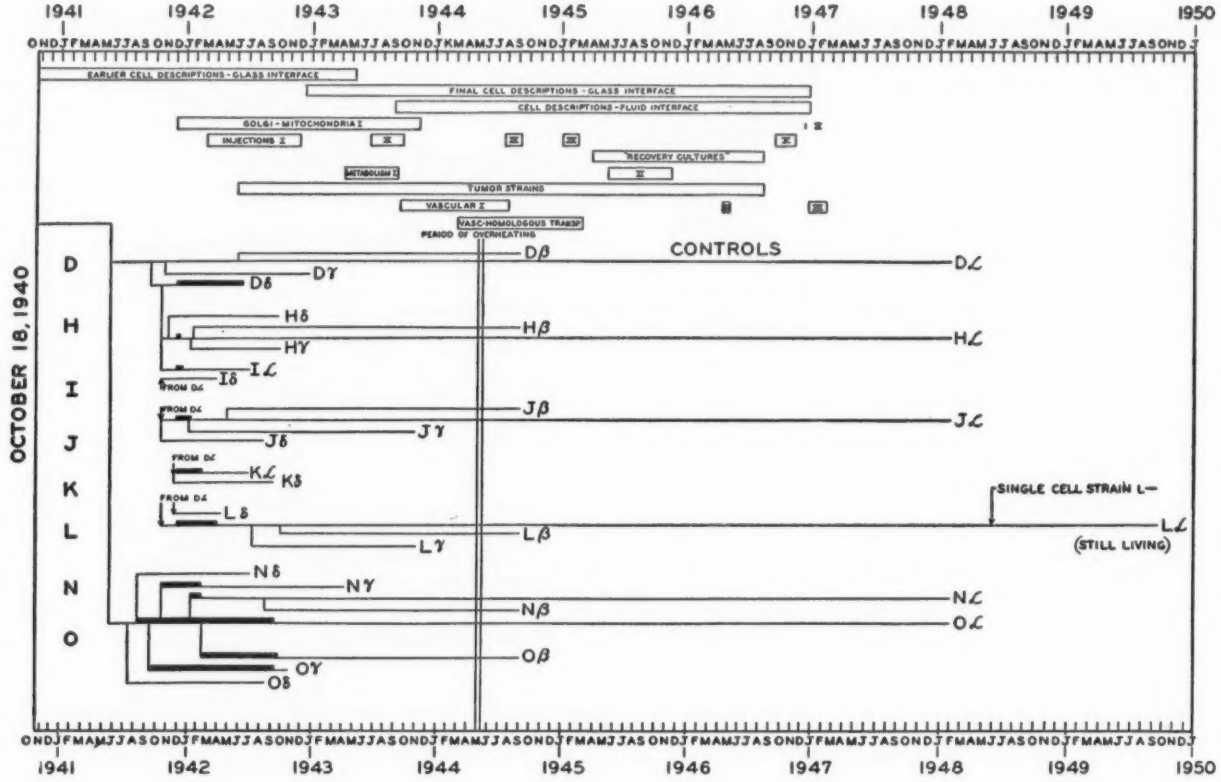
Strain H.—As compared with the cultures of the untreated parent strain at an earlier date, strain H cultures, originally treated for 6 days with carcinogen, in general showed excellent growth.

As compared with the cells of this early parent strain, the cells of strain H, between April 1942 and May 1943, showed a retraction of their slender terminal threadlike processes, a shortening of the cells, and a definitely closer lateral cohesion. This change in the cells was more pronounced than in cells of the D control strain, although like those of the D strain the cells of the H strain continued to be radially oriented relative to the explant. Cell size was uniform, and practically no extremely large cells were observed.

No further definite trend of change was recognized in this strain of cultures even though detailed examinations were made up through December 1946 (fig. 3), while less regular periodic visual examinations of the strain were made up through January 1948.

Strain J.—The cultures of strain J, treated originally with carcinogen for 32 days, showed growth consistently less luxuriant than did those of strains D, H, and L.

As compared with cultures of strains D and H, from December 1942 to June 1944, strain J cultures showed a greater tendency to assume more sheetlike shapes and to send out short lateral processes. Consequently the individual cells of strain J tended to more irregularly angular shapes, and showed less of a definite radially oriented major axis. This tendency


1941
1942
1943
1944
1945
1946
1947
1948
1949
1950
EARLIER CELL DESCRIPTIONS - GLASS INTERFACE
FINAL CELL DESCRIPTIONS - GLASS INTERFACE
CELL DESCRIPTIONS - FLUID INTERFACE
GOLGI - MITOCHONDRIA I
INJECTIONS I
RECOVERY CULTURES
METABOLISM II
TUMOR STRAINS
VASCULAR I
VASC-HOMOLOGOUS TRANSP
PERIOD OF OVERHEATING
OCTOBER 18, 1940
D
H
I
J
K
L
N
O
CONTROLS
Dβ
Dδ
Dγ
DL
Hδ
Hβ
Hγ
HL
Iδ
IL
FROM DL
FROM DL
Jβ
Jγ
Jδ
JL
FROM DL
KL
Kδ
FROM DL
Lδ
Lβ
Lγ
SINGLE CELL STRAIN L—
LL
(STILL LIVING)
Nδ
Nγ
Nβ
NL
OL
Oβ
Oγ
Oδ

TEXT-FIGURE 1.—Chart to show the relationships of the more important ancestral lines of each of the cell strains studied, and the chronological relationships of different phases of the research presented in this and other articles of the series.

The time during which each treated culture strain or line was subjected to 20-methylcholanthrene is shown by a very heavy horizontal black line. Each cell strain is designated by a letter while subordinate lines are indicated by Greek letters. In all instances the α line of each strain carried for any length of time is that which was maintained up to February 1948. Strain L is still being maintained in culture in May 1950, while from it, in June 1948, a single cell was explanted and grown into a pure line culture, which is also still being maintained. All β line cultures were lost by bacterial infection September 8, 1944, while all γ, δ and ϵ cultures were closed earlier for various technical reasons.

The period shown by the horizontal bar marked "earlier cell descriptions" is that covered by the cell descriptions previously reported (*1*). The next horizontal bar from the top indicates the period covered by the present detailed description of the cells at the glass interface of the culture, while the third bar indicates the period of study of the cells at the fluid interface (*2*). The bar marked "Golgi-mitochondria I" shows the period of time covered in the previous paper on the Golgi and mitochondria (*5*), while a short period of study in late 1946, not yet reported, is also indicated. The bars labeled "Injections I–V" indicate the periods of 5 different groups of injections of tissue cultures into mice; of these only the early results have been reported (*6*). The period of regrowth in culture of cells from the originated tumor strains, a study as yet unreported, is shown by the bar marked "Recovery Cultures," while the periods of metabolic studies of the originated tumor strains is indicated by bars marked "Metabolism I and II," the first of which has been reported (*7*). Subinoculations of originated tumor strains were carried as shown by the bar marked "Tumor Strains." The behavior of these subinoculations will be described in detail in a later article. The bars marked "Vascular I, II, and III" indicate the periods during which studies were carried out on the vascular reaction resulting from injection of the tissue cultures into mice. The bar marked "Vascular-homologous transplant" indicates the period of studies on vascular reaction resulting from injection of tissue of the various strains of tumors into mice. These studies are still unreported.

often resulted in production of extensive, almost uninterrupted, thin cell sheets (fig. 4). Often, however, lateral cell cohesion along the whole periphery of the cell did not occur, but instead cells cohered by means of short terminal or lateral processes into irregular strands. At the edge of the culture, such strands of flattened cells grew out into the clot, and then, apparently by cohesion along a lateral or angular edge, and by migration along a lateral or angular cell axis, the strand would start to grow at an angle to its original axis (fig. 5) and would continue this angular migration and finally, uniting with other angularly oriented strands, would enclose an irregular area of clot. As the cell sheet advanced these enclosed areas of clot remained for some time as irregular fenestrations within the cell sheet.[5] The formation of such fenestrations in this strain was often so extensive that the whole outer zone of the cell sheet assumed a "lacelike" [6] appearance (fig. 6). In such severely fenestrated sheets the angular paths of cell migration further disturbed the radial orientation of the cells of the sheet relative to the explant.

From about July 1944 on, this strain showed less tendency to form sheets. The lateral cohesion of the cells appeared substantially lessened, with the result that the sheet became looser, and its fenestrations less complete. Consequently the sheet broke up peripherally into cell strands, and even in these strands the lateral cohesion of the cells was reduced so that the strands were often loose and ill defined (fig. 7). In cultures that showed an extreme stage of the alteration (fig. 8) the periphery of the culture practically dissociated into individual, loose, flattened, spindle-shaped, or more often polygonal or angular disoriented cells. Frequently cells were of very irregular shapes, and in some cultures many cells showed extreme rippling of their periphery. In these later cultures there was in instances a suggestion of increased cell granulation. In areas there were appreciable amounts of cellular debris, indicating cell distintegration, although in these same areas, mitoses continued to occur.

Data do not allow following these cell changes beyond December 1946. The strain was closed in February 1948.

Strain L.—Growth of strain L culture, originally treated 111 days with carcinogen, was extremely luxuriant.

As seen in December 1942 to March 1943, the cells of this strain appeared appreciably more altered morphologically than those of strain J, in that they showed a more extreme shortening of the cells with a tendency toward more sheetlike or rounded shapes, and with the frequent production of cells larger than normal size. The cell sheet at the glass interface of strain L culture was less fenestrated than was that of the "lacelike" design of strain J. Where fenestrations did occur they were large and more irregular (fig. 9). Cell strands were usually slender (fig. 10), and made up of thin, flattened sheetlike or occasionally more rounded or

[5] This mechanism of fenestration in a cell sheet is entirely different from that so frequently seen resulting from liquefaction of the culture matrix. In this latter case liquefaction-perforation of the plasma clot is followed by subsequent shrinkage of the surrounding plasma and its contained cells away from the perforated area.

[6] This "lacelike" design of the cell sheet was mentioned in the original description (*1*) of this strain during the interval from September 18, 1942 to May 1943, but was not included in the illustrations shown for this period.

spherical shaped cells. Where the cell sheet showed little or no fenestration the cells were very closely coherent laterally, usually less elongate, and more sheetlike than in strain J. They were, in general, less definitely radially oriented than in the unfenestrated area of strain J culture. Such a continuous sheet is shown in figure 11. The same rounding of the cells seen in the fenestrated sheets was often seen in the continuous sheets.

While more numerous photographic records allow some amplification of details of the design of this strain over that given in the earlier paper (*1*), the morphology of the strain appeared stable, within the period of study from December 1942 to December 1946. At the present time (June 1949) this cell strain is still growing luxuriantly, and still appears essentially stable in its morphology. As previously reported it has been satisfactorily transferred from plasma to cellophane substrate (*8*). From this strain a single cell has been isolated (*9*), grown into a macroscopic culture, and is now being maintained in stock culture on cellophane. Even in this cell strain originated from one single cell, the strain continues to grow luxuriantly and to show a rather unusual range of cell size, with frequent production of cells much larger than normal (fig. 12).

Strain N.—The growth of strain N culture has in general been much inferior to that of strains D, H, and L. The cultures of strain N have also shown much greater tendency to early central necrosis, particularly at the glass interface. This necrosis has often extended out almost to the periphery of the culture. This poor growth continued up until the strain was closed on February 1948.

The cells of these cultures, as has been described, up to May 1943 showed a generally increased cytoplasmic granularity, an increased tendency toward rounded or spherical shapes and an increased tendency toward extreme lateral cohesion of the cells as compared with those of less altered strains D, H, J, and L.

Up through January 4, 1945, the cultures frequently showed the closely coherent, massive cell sheet previously described for cultures of strain O (*1*). In these living sheets, cell cohesion, both lateral and terminal, was so extreme that cell boundaries were not distinguishable. Examination of living cultures gave a substantially clearer picture of this lateral cohesion into massive sheets than was possible in fixed and stained preparations. Even with the most careful fixation (*10*) the cells often drew apart, while the accentuated staining of the nuclei tended to reduce emphasis on cell boundaries. The edges of these sheets often appeared relatively thick and in instances showed a finely ruffled or serrated edge as is illustrated in figure 13. In other instances the edge of the sheet was irregularly extended into short rods and irregular cell masses. Where these projections were less massive, the tendency of cells and cell clumps to form rounded or spheroidal masses was very distinct (fig. 14).

In instances the periphery of the culture was looser and broken up into projecting, often interconnected strands of rather elongated, flattened, or somewhat laterally rounded, granular cells, as shown in figure 15. In a few instances this interconnecting of cell strands resulted in producing

a very delicate network of cells. An extreme example of this is shown in figure 16, but even in such networks the cells were granular and tended toward laterally rounded or spherical shapes.

With the passage of time after removal from the carcinogen, the cultures of this strain N showed fewer closely coherent cell sheets. Instead, looser designs, such as those shown in figure 17, became more frequent. Although no extremely large cells were seen, the cells in such areas tended toward irregular and rounded shapes. The frequent presence of cell debris indicated an appreciable amount of cell disintegration. Data do not permit following the cell changes of strain N beyond December 1946, at which time the progressive morphologic changes described were still in progress. The strain was closed in February 1948.

Strain O.—The growth of the carcinogen-treated cultures of strain O was much inferior to that for cultures of strains D, H, and L, while the tendency to show early and extensive central necrosis, particularly of the cells at the glass interface, was even more severe than in strain N. The limited growth of these strain O cultures together with this persistent tendency to extensive central necrosis made this strain the most difficult of any of the series to maintain in culture. This poor growth continued up until the strain was closed in February 1948.

The cells at the glass interface of strain O cultures were rather uniform in size; only an occasional very large cell was observed (fig. 18). Like the cells of strain N, those of strain O showed a generally increased cytoplasmic granularity and a more extreme lateral cohesion of the cells than in strains D, H, J, and L. In these respects the cells of strain O were even more extreme than those of strain N. The tendency toward rounded or spheroidal cell shapes was quite as great as in strain N.

As with strain N, up to about June 1944, the lateral cohesion of the cells of these cultures was frequently extreme and resulted in the production of numerous, continuous, often relatively thick, cell sheets, as in figure 19. In some instances the edge of the cell sheet was broken up into irregular lobes or projections of rounded cell masses and rounded cells, as shown in figure 20.

As with strain N, even at the time of removal from the carcinogen September 16, 1942, some cultures showed looser cell sheets. With the passage of time the massive coherent cell sheets gradually disappeared from the cultures while the looser designs, such as those shown in figures 21 and 22, became dominant. In cultures, even as late as December 19, 1946, however, cell cohesion was still exaggerated (fig. 22) while the granularity of the cells persisted.

Data do not allow following detailed cell changes beyond December 1946. No further marked changes were observed in the morphology of the cells even in February 1948, when the strain was closed.

An attempt was made for several transfer generations to propagate specially cohesive cultures of strains N and O. In the several generations of cultures studied there was not much consistency in the reappearance

of the massive sheet design in consecutive transfer generations. Sometimes, cultures that showed no massive cell sheets in one generation, in the next transfer generation showed such sheets. In other instances cultures that originally grew out as a massive sheet of cells, after 10 or 15 days showed dissociation into looser cell designs. In still other instances a massive cell sheet migrated down through the plasma, possibly due to some local defect in the plasma clot, reached the fluid interface of the culture, and there assumed the loose design of growth characteristically seen at the fluid interface. From the changes observed it appeared that while closely coherent and massive cell sheets were characteristic of strain N and O, these sheets could dissociate into looser cell designs, or looser cell designs in these strains could in instances associate into more closely coherent sheets.

DISCUSSION

In a previous paper (*1*) characteristic cell designs at the glass interface of each of the six strains of cultures were described up to May 15, 1943. This previous paper included a description of cell change up to approximately 1 year before the accidental overheating of the cultures previously described (*2*). Subsequent to this overheating, cultures of strains D, H, and L showed a rapid return to the characteristic rates of growth and to the designs of morphology which they had shown previous to overheating. The later growth of cultures of these three strains continued excellent and comparable to that shown before the overheating, while the general morphology at the glass interface of each of these three strains continued unchanged and stable during the remainder of the study. It appears then that for cell strains D, H, and L, at least, the overheating may be dismissed as having no significant role in influencing the culture characteristics reported.

Subsequent to the overheating the culture of strains J, N, and O continued to show further progressive alteration in cell design. While it is possible that the accidental overheating that had occurred may have had some role in causing the later changes in these three cell strains, in the case of strains N and O at least this seems improbable, since photographs suggest that these later changes in cells had already begun prior to the period of overheating.

Data available do not allow definite conclusion as to what other factors may have been responsible for the later progressions of change in the cells of strains J, N, and O. With current tissue-culture methods, as used in this study, cultures carried over an extended interval of time must ultimately consist only of those cell types able to survive under the culture conditions used. The long continued, progressive change observed in cultures of strains J, N, and O, after removal from carcinogen, may well have been due to just such gradual overgrowth of the cultures by cell types most readily able to survive under the culture conditions used. It is possible, however, that in these three strains certain cell changes induced during the carcinogen treatment were unstable and either sub-

sided or underwent further alteration after removal of the cells from the carcinogen.

Considering further the permanence of the cell changes induced in each of the six cell strains, untreated strain D and treated strain H, when examined in late 1946, approximately 5 years after removal of strain H from carcinogen, still showed altered morphologies indistinguishable from those shown in December 1942. Less detailed records indicate the morphology of each of these strains as stable until the time they were closed in February 1948.

The cells of treated strain J, in December 1946, nearly 5 years after its removal from carcinogen, had lost much of their lateral cohesiveness at the glass interface of the culture. However, even when the strain was closed in 1948, it still appeared greatly altered as compared with the parent strain. Compared with the cells of strain H, those of strain J were more flattened, sheetlike, and more randomly oriented relative to the explant. The growth of these strain J cultures continued inferior to that of either strain D or strain H.

Treated strain L, in December 1946, approximately 4¾ years after removal from the carcinogen, still showed an altered morphology indistinguishable from that shown in December 1942. This strain, characterized by numerous abnormally large cells, particularly at the fluid interface of the cultures, and by greatly increased cohesion of the cells at the glass interface, was definitely more altered from the parent strain than were strains D, H, or J. Even after this strain was transferred to cellophane substrate and was propagated from a single isolated cell, growth of the cultures originated from the single cell continued to show a very similar characteristic cell design and an unusual number of abnormally large cells, 7 years after the removal of this strain from carcinogen.

Treated strains N and O, at December 1946, 4¾ and 4½ years after removal from the carcinogen respectively, still showed the poorest and most unthrifty growth of any of the strains, and were still the most difficult strains to maintain in culture. They still showed greatly retarded culture growth with severe central necrosis of the culture. These growth characteristics continued up until the strains were closed in February 1948, 5½ years after removal of strain O from the carcinogen. Detailed records of cell descriptions showed that increased cell cohesion at the glass interface, the lateral rounding of the cells at fluid interface of strain N, and the characteristic appearance of shrunken cells at the fluid interface of strain O all continued as long as such records were carried, that is up through December 1946. Even during later examinations of these strains they were considered as definitely more altered from the parent strain than were any of the preceding strains, while strain O was considered as more altered than strain N.

It appears then that even more than 4½ to 7½ years after removal of the treated strains from the carcinogen, the progressive, gradual, cumulative changes induced in the cells during their exposure to carcinogen have to an extraordinary degree remained stable and permanent. How stable they

were may be further emphasized. In strain H, allowing most conservatively 5 days for each intermitotic interval, this cell strain showed no recognized change in its characteristic morphology in more than 380 consecutive cell generations since the photographic record of this strain, in late 1942, and up to the time the strain was closed in 1948. In the more than 7 years that strain L has been carried, it must have gone through many generations in excess of 510.

Including earlier cell strains studied in this investigation (*11*) the characteristic cell changes seen in the cells of cultures treated with 20-methylcholanthrene has now been reproduced in four independent cell strains each taken from a different mouse of the C_3H strain. In each instance, the change, beyond the limited extent of change seen in the controls, has occurred only under the action of this carcinogen, while the degree of change of the cells has been closely correlated with the duration of treatment with the carcinogen. Finally, the more altered cell strains have appeared to pass progressively through the less altered stages described for cells treated for short intervals with the carcinogen. These observations all indicate that the change in the treated cells, beyond that stage reached by the controls, was closely and directly correlated with the action of 20-methycholanthrene on the cells. This group of cell changes has now been reproduced in more or less detail in four independent strains of fibroblasts from the C_3H strain mouse. It appears then that under the experimental conditions used, the progressive, morphologic cell change described is the general type of reproducible characteristic reaction of strains of fibroblasts from the subcutaneous and muscular tissue of the C_3H mouse to this carcinogen.

That this type of reaction seen in the treated culture is induced by other carcinogenic agents or agencies is also indicated by the observation of Jacoby (*12*) who reported a closely comparable design of architecture in cultures of dibenzanthracene-induced sarcoma from the mouse. A limited group of observations carried out in our laboratory [7] demonstrated a similar shortening and lateral cohesion of the cells in cultures made from a spontaneous sarcoma from the subcutaneous tissue of a C_3H strain mouse.

With respect to the cause of the original limited cell change observed in the presumably untreated control strain D, data available indicate the change in this cell strain as entirely comparable in nature, but more limited in degree, to the changes shown by the strains deliberately treated with the carcinogen. At the present time the possibility of a trace contamination of this D strain with the 20-methylcholanthrene used on the treated cultures cannot be entirely excluded even in view of the care taken in handling the material. However, the possibility certainly exists that this change in the cells was induced by an entirely different agent or agency, which acted to induce the same ultimate type, though more limited degree, of stable change in the cells. A definite conclusion as to what caused the change in these presumably untreated control cells is therefore not warranted by data now available.

[7] Unpublished observation.

As stated, it appeared that the change in the previously untreated control cells was of the same type, but was more limited in degree than that seen in the treated cells. If we assume that this spontaneous limited change in the control cultures was due to a trace contamination with 20-methylcholanthrene, it appears then that the 20-methylcholanthrene as used under the defined experimental conditions acted as an agent directly or indirectly able to induce a relatively permanent morphologic and physiologic change in the cells. If, however, the changes in the control cells were either "spontaneous" in origin or due to some agent or agency, other than 20-methylcholanthrene, then 20-methylcholanthrene must be considered as either an additional transforming agent or an agent greatly accelerating an already occurring transformation of the cells.

At the present state of our knowledge none of the morphologic changes observed can be set up as an index either for the conclusive recognition of the malignant fibroblast or as an index for decision as to when a "normal" fibroblast becomes able to give rise to sarcoma on injection into animals of its own strain. By the methods of study employed, the morphologic transition from the "normal" fibroblast to that of strain D (as shown in figs. 1 and 2) was so gradual and in many instances so ill-defined as to be uncertain of recognition. However, certain of the later changes observed in fibroblasts treated with 20-methylcholanthrene were so sharp and easily recognizable as to be extremely useful as indices of change in the study of the progressive transition of the cell. Such clearly defined changes as the progressive shortening of the cell with loss of its slender terminal processes and with lateral cohesion into sheets and massive sheets, the production of numerous "giant cells" in the fluid interface at stage L, and the lateral rounding of the cells with disappearance of giant cells in the fluid interface at stage N appear to be easily recognizable and useful indices.

The control of experimental conditions prevailing in the cultures and the methods of study of the cultures which could practically be used over such a long period of investigation have been all too limited. With the development of new methods of study of tissue cultures of pure cell strain over long intervals of time, under better controlled conditions, and with the development of methods that allow a more accurate following of changes in cell migration, cell proliferation and cell transformation, a restudy by tissue culture methods of the action of a chemical carcinogen on a pure cell strain would appear well justified.

SUMMARY AND CONCLUSIONS

1. A chart is presented to correlate more closely the chronological relationships of the various studies presented and the relationship of the various cell strains and subordinate lines studied during the more than 8 years this series of investigations has been in progress.

2. The six cell strains studied in detail include one untreated control strain (D) and the 5 strains H, J, L, N, and O, treated with 20-methylcholanthrene for 6, 32, 111, 184, and 406 days respectively. These strains

have been continued as cultures growing in an entirely heterologous medium of chicken plasma, horse serum and chick embryo extract for more than 5 years after the removal of the last of the treated strains from carcinogen. One of these strains (strain L) is still being carried. The remainder of the strains were closed in February 1948.

3. Supplementing the earlier description of the manner of growth of the cultures and the design of cell growth at the glass interface, as described up to May 1943, detailed description of the growth of these cell strains continued up to December 19, 1946, and is supplemented by less detailed observations up until the time the strains were closed. In general, strains D and L grew luxuriantly, with H less luxuriantly, J even less. Strains N and O were difficult to handle because of poor growth and early necrosis.

4. Detailed descriptions are given supplementing those previously given of the cell morphology at the glass interface. Subsequent to December 1942, strains D, H, and L showed no appreciable change in morphology throughout the whole period of study. Strains J, N, and O showed some loosening of the cell sheet, but preserved unchanged other characteristic changes that had been induced in them during carcinogen treatment. In the years since the last of the treated strains was removed from carcinogen, they have in general shown an extraordinary stability in maintaining their altered characteristics. This may be emphasized, for instance, in the case of strain H. Allowing, most conservatively, 5 days for each intermitotic interval the cells of strain H showed no recognizable change in more than 380 generations up to the time the strain was closed in 1948.

5. As compared with the fibroblasts of the parent strain, from which all strains arose, the degree of alteration in cell design at the glass interface of the cultures of these six strains appeared to be directly correlated with the duration of exposure of the cells to 20-methylcholanthrene. All treated strains showed greater alterations than did the presumably untreated D control strain.

6. There are no data from these studies that warrant considering the changes in the presumably untreated D control strain as essentially different in anything except degree from those shown by the cell strains deliberately treated with the carcinogen.

7. This general type of progressive morphologic cell change described as associated with treatment of the cells with 20-methylcholanthrene, has now been produced in four independent cell strains, each from a different C_3H strain mouse. It appears that these changes described are the characteristic reproducible changes associated with the action of this carcinogen on the subcutaneous fibroblast of the C_3H strain mouse. Since Jacoby describes apparently comparable changes associated with the *in vivo* action of dibenzanthracene in mice, the changes described in this article are apparently not specific for 20-methylcholanthrene but are apparently associated with the action of at least one other chemical carcinogen as well.

8. As studied *in vitro* under the experimental conditions used, it appears that 20-methylcholanthrene must be considered as an agent having a definite role in inducing the cell changes described in the treated strains, or as an agent tremendously accelerating such already occurring changes.

REFERENCES

(*1*) EARLE, W. R.: Production of malignancy in vitro; mouse fibroblast cultures and changes seen in living cells. J. Nat. Cancer Inst. 4: 165–212 (1943).

(*2*) EARLE, W. R., SCHILLING, E. R., and SHELTON, EMMA: Production of malignancy in vitro. IX. Description of cells at the fluid interface of the culture. J. Nat. Cancer Inst. 10: 865–882 (1950).

(*3*) EARLE, W. R.: Production of malignancy in vitro; method of cleaning glassware. J. Nat. Cancer Inst. 4: 131–133 (1943).

(*4*) ———: Production of malignancy in vitro; photomicrography equipment. J. Nat. Cancer Inst. 4: 135–145 (1943).

(*5*) DALTON, A. J., and EARLE, W. R.: Production of malignancy in vitro; observations on mitochondria and Golgi material. J. Nat. Cancer Inst. 4: 539–554 (1944).

(*6*) EARLE, W. R. and NETTLESHIP, A.: Production of malignancy in vitro; results of injections of cultures into mice. J. Nat. Cancer Inst. 4: 213–227 (1943).

(*7*) BURK, D., EARLE, W. R., and WINZLER, R. J.: Production of malignancy in vitro; metabolism and biotin vitamer content of tumors produced. J. Nat. Cancer Inst. 4: 363–372 (1944).

(*8*) EVANS, V. J., and EARLE, W. R.: Use of perforated cellophane for growth of cells in tissue culture. J. Nat. Cancer Inst. 8: 103–119 (1947).

(*9*) SANFORD, K. K., EARLE, W. R., and LIKELY, G. D.: The growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst. 9: 229–246 (1948).

(*10*) EARLE, W. R.: Procedure for fixation, staining, and mounting of whole mounts from tissue cultures grown in Carrel D 3.5 flasks. J. Nat. Cancer Inst. 8: 83–89 (1947).

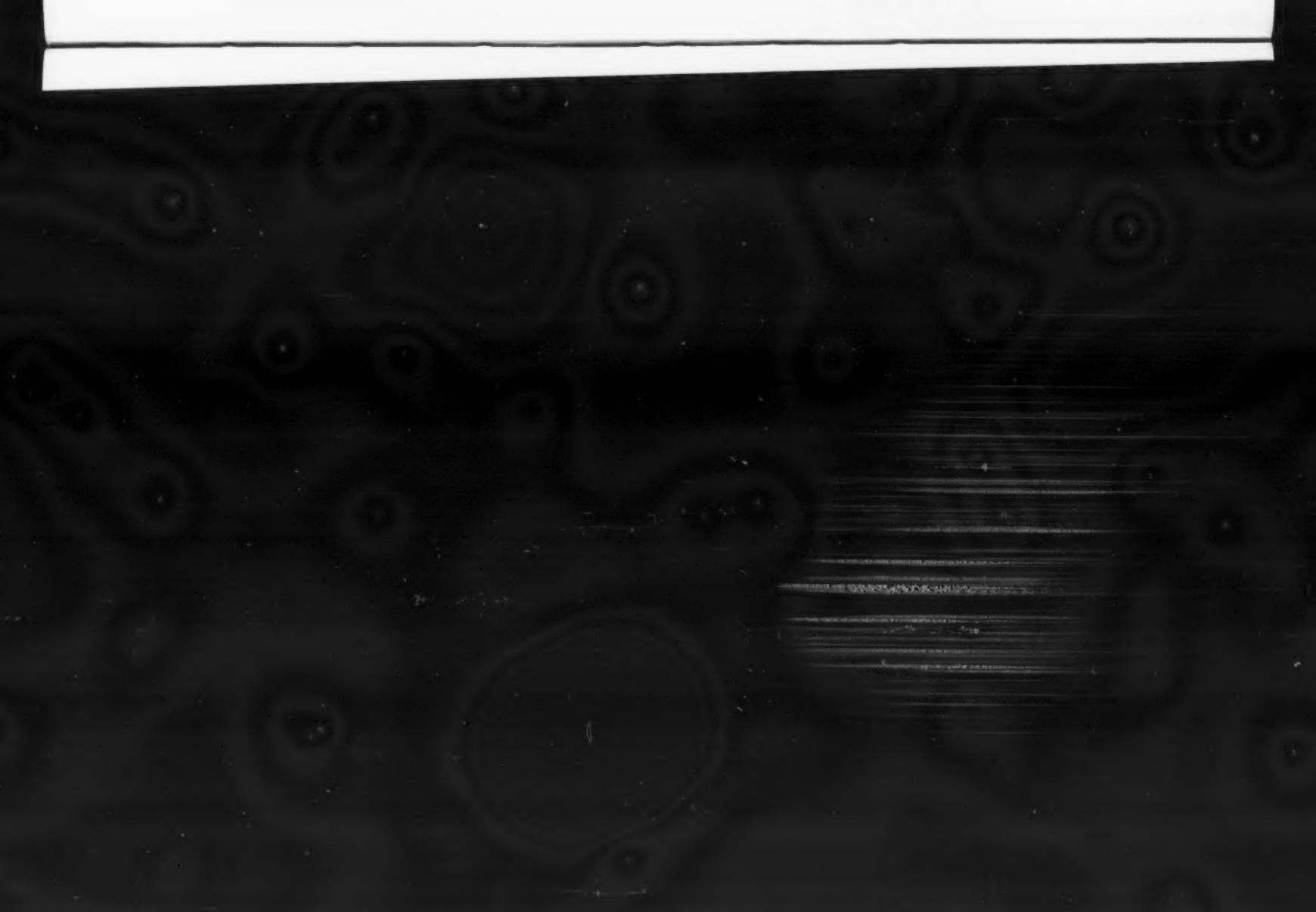
(*11*) EARLE, W. R., and VOEGTLIN, C.: Further study of mode of action of methylcholanthrene on normal tissue cultures. Pub. Health Rep. 55: 303–322 (1940).

(*12*) JACOBY, F.: Long-term cultivation in vitro of a dibenzanthracene mouse sarcoma. Nature, London 152: 299 (1943).

PLATE 166

FIGURE 1.—Cells from a strain D culture, α line, January 4, 1945, 12 days after planting. Note the formation of loose cell strands. × 200.

FIGURE 2.—Cells from a strain D culture, α line August 1, 1946, 10 days after planting. Note the extremely loose cell strands. × 200.



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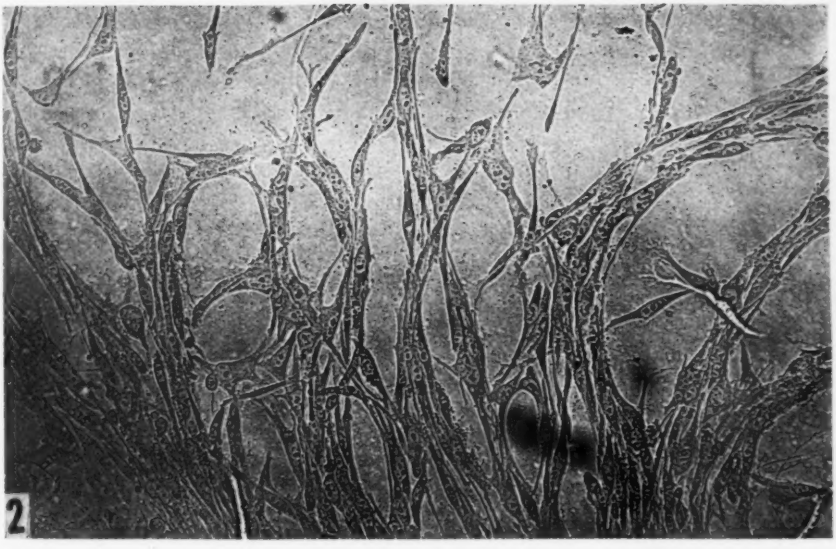
2

PLATE 167

FIGURE 3.—Cells of strain H culture January 4, 1945, 12 days after planting. × 200.

FIGURE 4.—Cells from a strain J culture November 3, 1943, 12 days after planting. This area of cells shows sheetlike shapes and lateral cohesion rather extreme for strain J, but often seen in strain L cultures. × 200.

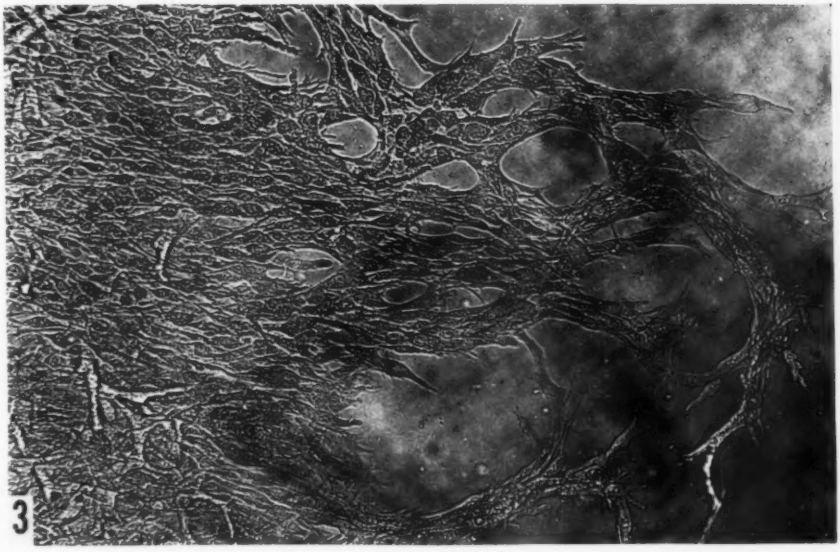
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PLATE 168

FIGURE 5.—Cells at the glass interface of a strain J culture October 1, 1943, 11 days after explantation. Note how the cells tend to migrate along angular axes, producing looping strands of cells that reconnect and enclose islands of clot. × 200.

FIGURE 6.—Cells of a strain J culture, January 6, 1944, 9 days after explantation. This area shows many enclosed islands of clot. This is typical of the "lacelike" design of growth at the glass interface of this J strain. × 200.

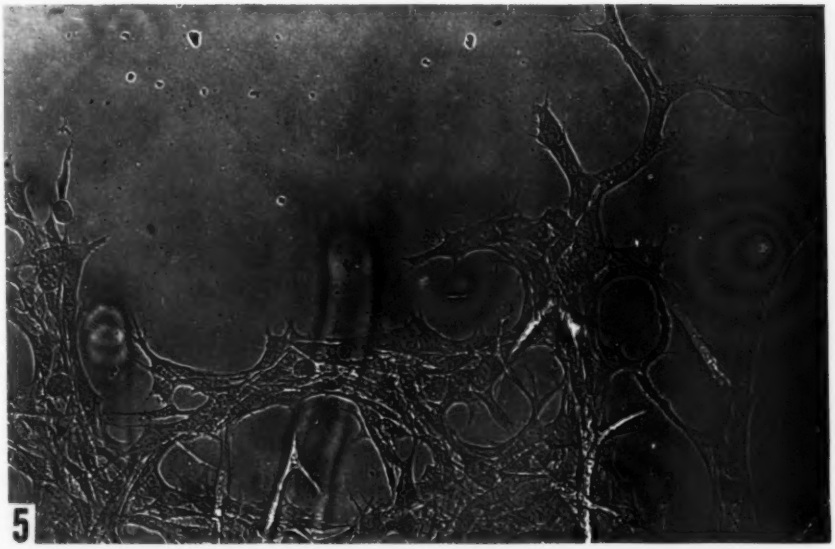
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PLATE 169

FIGURE 7.—Cells of a strain J culture December 27, 1944, 9 days after explantation. Note how loose and ill defined the cell strands have become. × 200.

FIGURE 8.—Cells of a strain J culture, September 26, 1946, 9 days after planting. Note the extreme looseness of the cell sheet and the increasingly random orientation of the cells as compared with earlier figures of the same strain. × 200.

7

8

PLATE 170

FIGURE 9.—Cells of a strain L culture September 26, 1946, 10 days after planting, to show typical fenestrated design. Note the increased tendency for the cells to be broader, more sheetlike, or in instances, to assume more rounded shapes than in strain J. × 200.

FIGURE 10.—Cells of a strain L culture February 1, 1945, 11 days after explantation. Note the cell strands made up of cells closely coherent laterally; note also the extremely large mononuclear cell in mitosis. × 200.

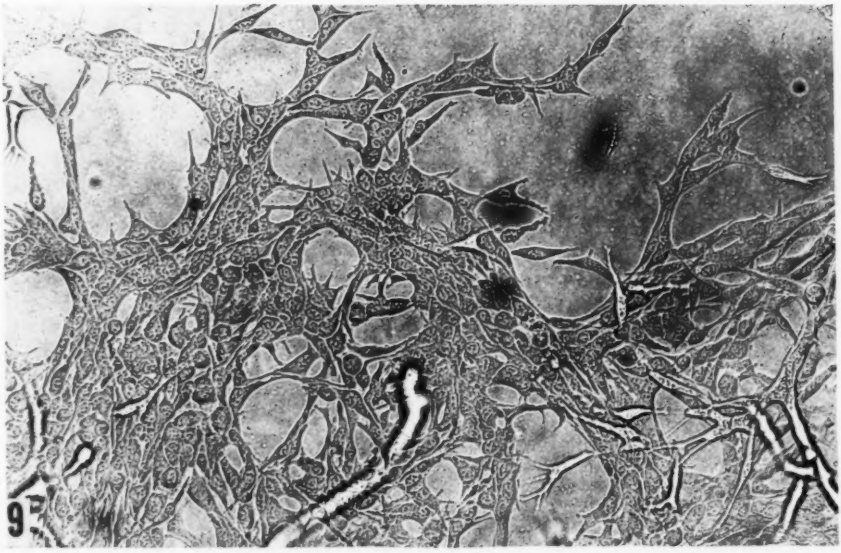
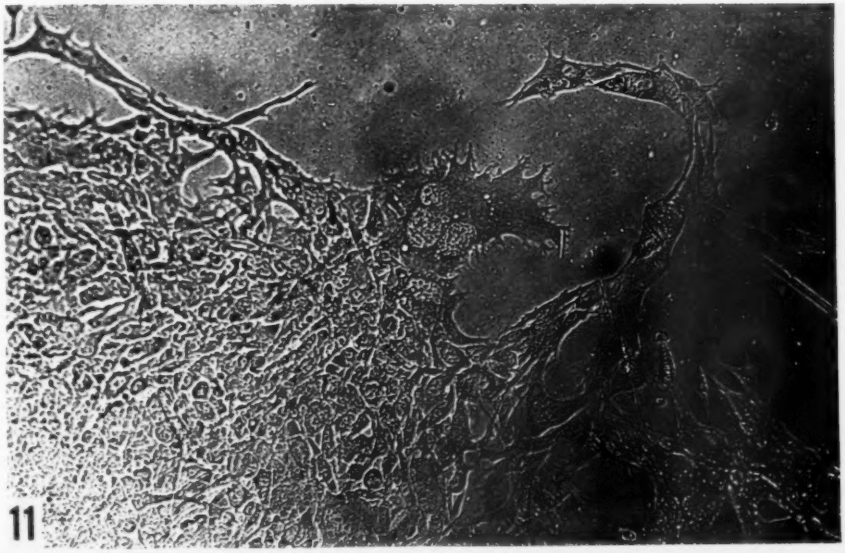


PLATE 171

FIGURE 11.—Cells of strain L culture December 29, 1944, 10 days after planting. Note the closely coherent cell structure of the cultures as compared with that of less altered strains. Note also the extremely large cell with three nuclei. × 200.

FIGURE 12.—Cells of strain L culture, photographed April 7, 1949, growing under perforated cellophane, 16 days after planting. Note variability of cell size. In such uncrowded cellophane cultures of this strain planted from a cell suspension, the cells often appear less laterally coherent than in plasma cultures that have grown from a central explant. A part of one of the perforations in the cellophane is shown at the left. This strain of cells was originated from a single cell culture. × 200.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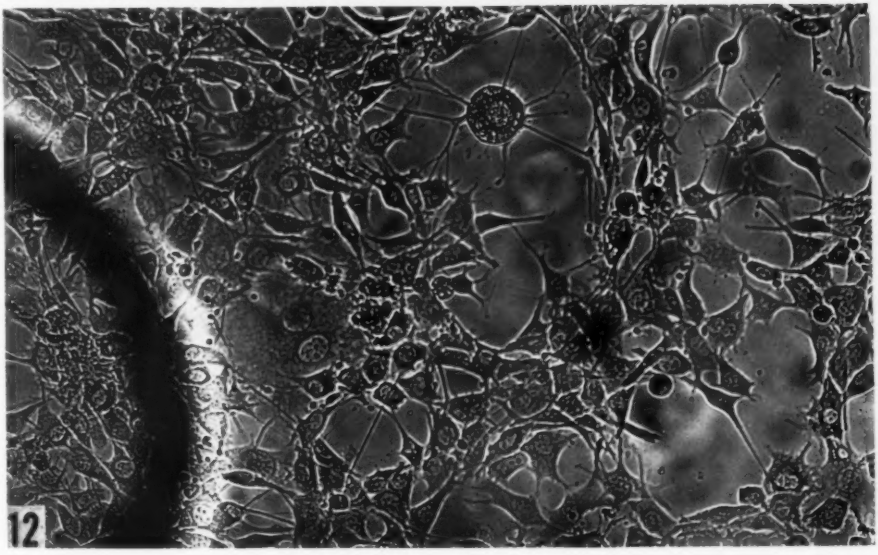
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PLATE 172

FIGURE 13.—Cells of a strain N culture September 4, 1943, 11 days after planting. Note the extremely close lateral cohesion of the cells to form such a massive cell sheet that even at its edge, lateral cell boundaries cannot be distinguished. × 200.

FIGURE 14.—Cells of a strain N culture September 4, 1943, 11 days after planting. The granular cells tend to be discretely rounded or to form rounded masses of cells at the edge of the culture. × 200.

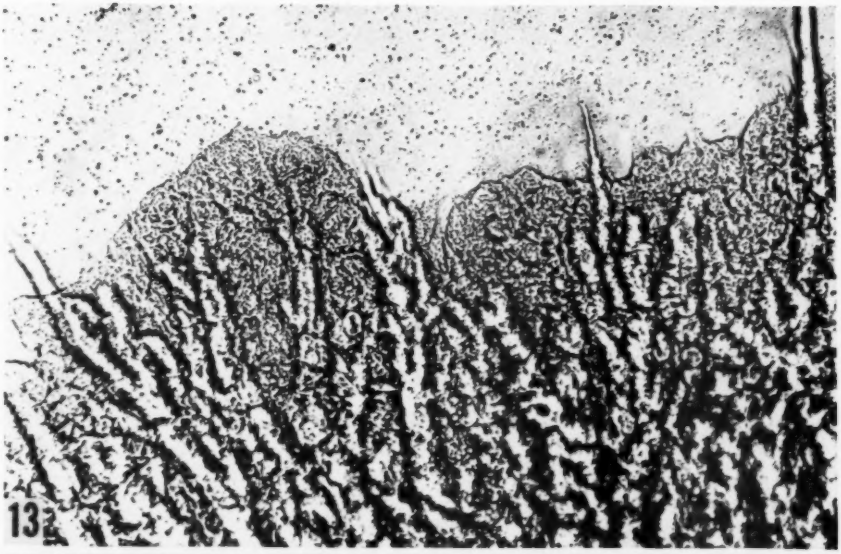
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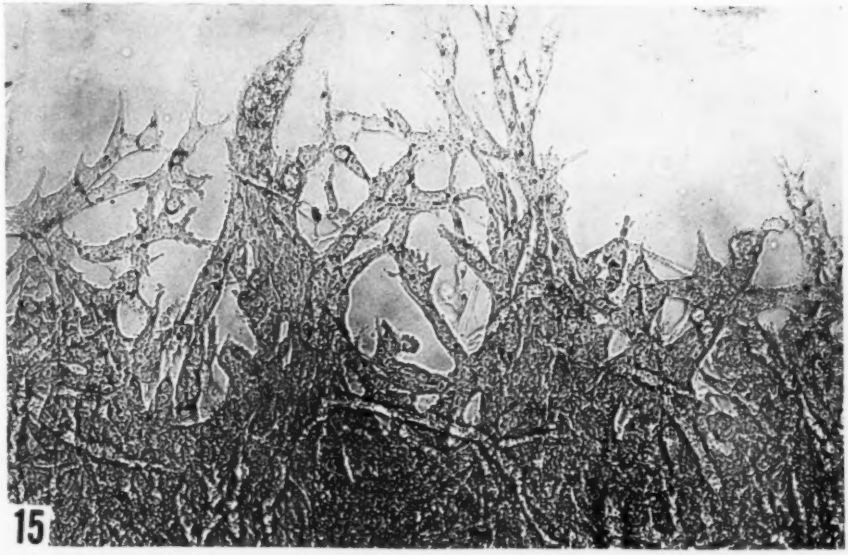
PLATE 173

FIGURE 15.—Cells of a strain X culture, January 6, 1944, 9 days after planting, to show production of cell strands. Note the ruffling of the cell lateral edges. × 200.

FIGURE 16.—Cells of a strain X culture September 4, 1943, 11 days after planting, to show a delicate network design in which individual cells often show tendencies to round. × 200.



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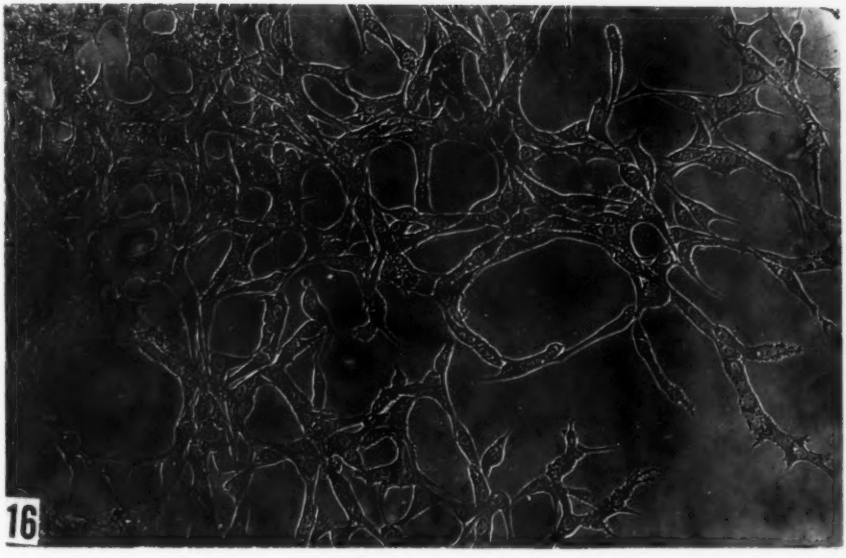

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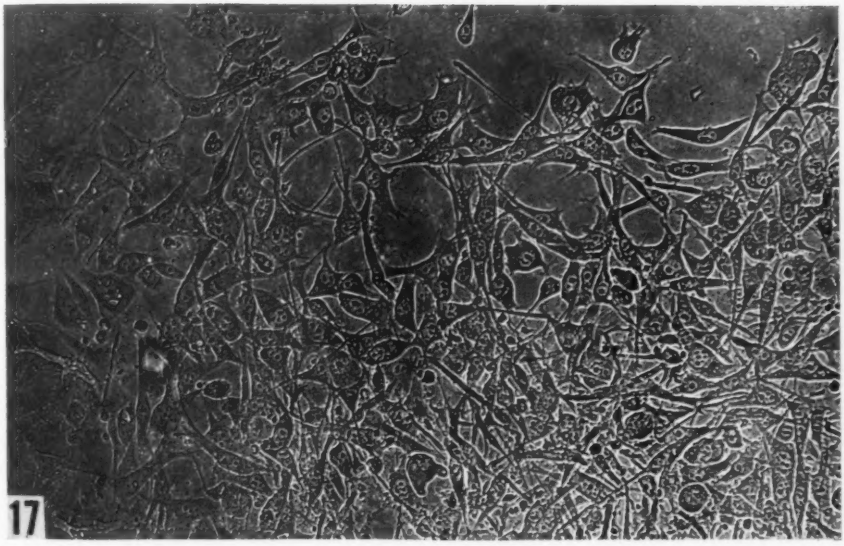
PLATE 174

FIGURE 17.—Cells of a strain N culture, September 26, 1946, 9 days after planting. Note the extremely irregular and disoriented cell shapes, some tendency toward cell rounding, and suggestion of cell disintegration. × 200.

FIGURE 18.—Cells of a strain O culture December 19, 1946, 8 days after planting. This shows one of the relatively few extremely large granular cells seen in strain O cultures. × 200.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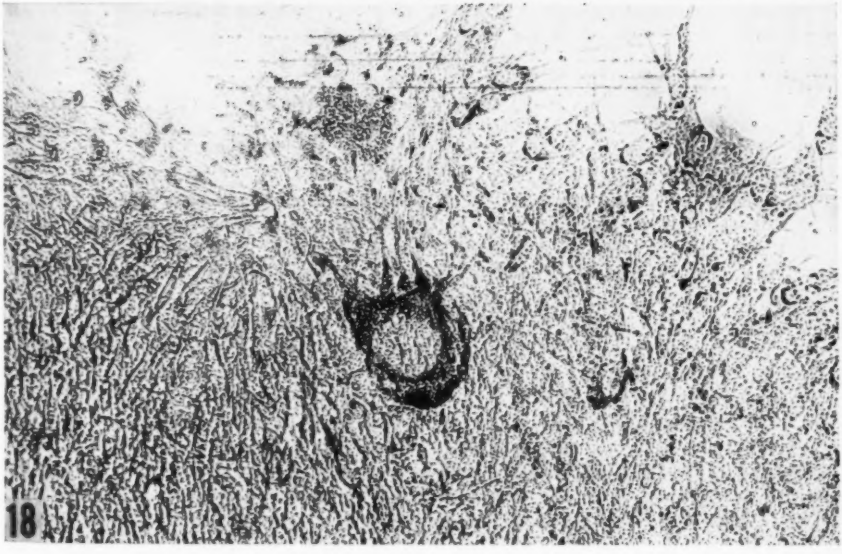
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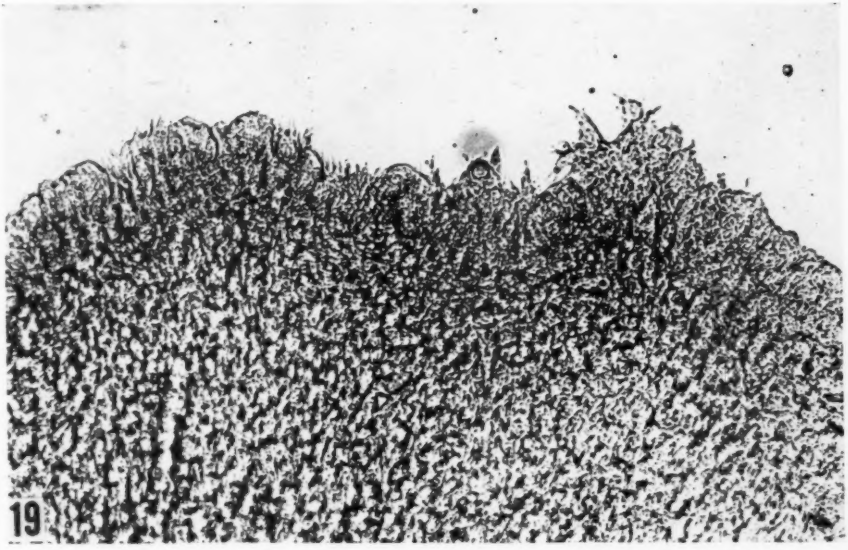
PLATE 175

FIGURE 19.—Cells of a strain O culture October 1, 1943, 11 days after planting, to show massive cell sheet design. × 200.

FIGURE 20.—Cells of a strain O culture September 4, 1943, 11 days after planting, to show less massive cell sheet with marked tendency of cells and cell masses to assume spherical shapes. × 200.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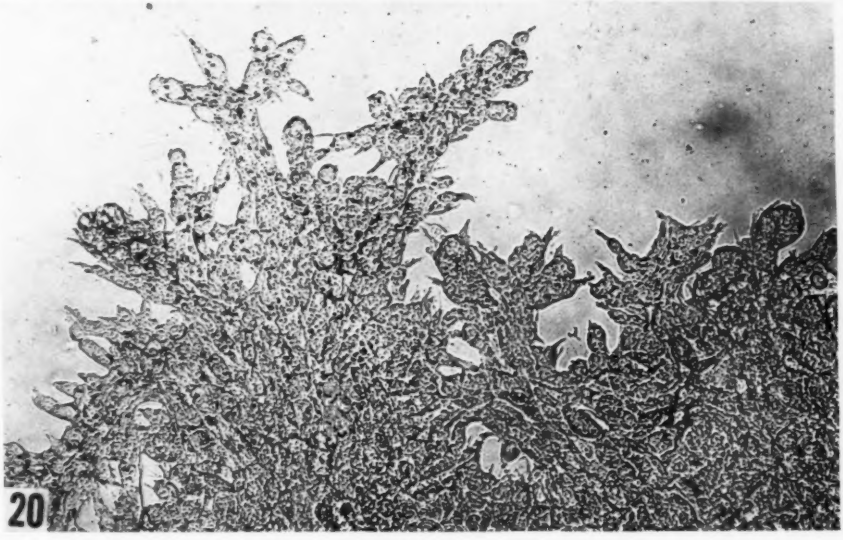
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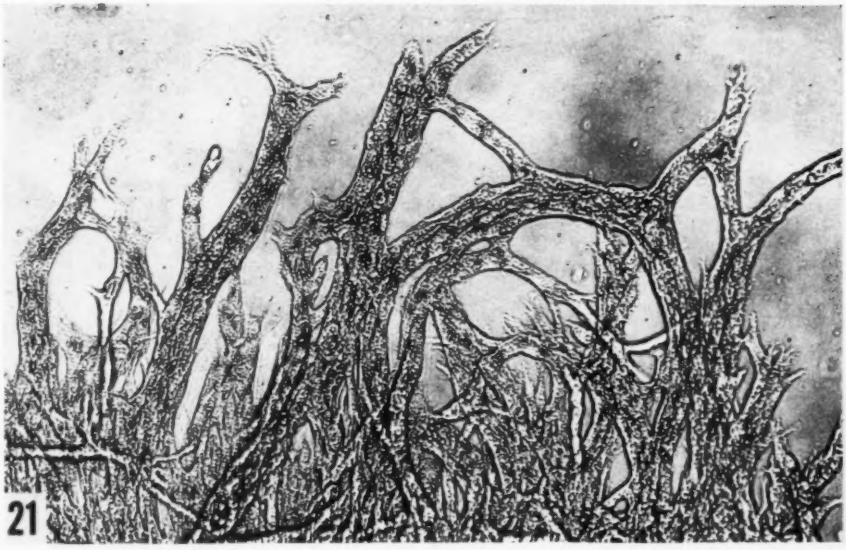
PLATE 176

FIGURE 21.—Cells at the glass interface of a strain O culture, January 4, 1945, 12 days after planting. The rather rodlike cell strand is characteristic of certain O strain cultures. × 200.

FIGURE 22.—Cells of a strain O culture December 19, 1946, 8 days after planting. Note the persistence of cell shortening, lateral cohesion of the cells, and severe granulation. × 200.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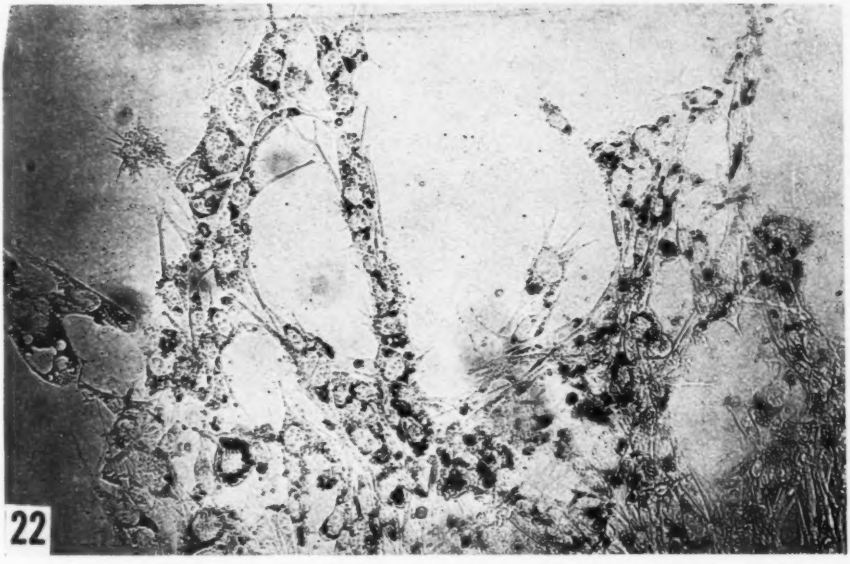
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PRODUCTION OF MALIGNANCY *IN VITRO*. XI. FURTHER RESULTS FROM REINJECTION OF *IN VITRO* CELL STRAINS INTO STRAIN C_3H MICE [1]

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INTRODUCTION

In order to test the ability of the six tissue-culture strains of mouse fibroblasts designated D (control), H, J, L, N, and O (*1*, *2*, *3*, *4*, *5*) to give rise to tumors *in vivo*, various cultures from each of the cell strains have been injected into mice of the C_3H strain, this being the same strain from which the original tissue had been taken. The results of early injections of these cells have been previously described (*6*, *7*). The present paper gives results from four later groups of injections of these cell strains up through November 1946.

MATERIALS AND METHODS

The technique used in the earlier injections has already been described (*6*). The technique used in the later injections was similar to that described in those experiments where tuberculin syringes were used. In all the later injections one whole culture 28 to 30 days of age was used for each injection. Each culture was loaded through a small funnel (text-fig. 1) into the barrel of a 1 cc. tuberculin syringe, previously fitted with a 1½-inch 18-gauge needle and sterilized by dry heat. A separate funnel and syringe were used for each culture.

The hair of each mouse to be injected was clipped over the rear part of the back in an area extending on both sides of the spine. The clipped area was then swabbed with 1:1000 tincture of metaphen, and most of this metaphen was then wiped off with a swab dipped in absolute alcohol. The injection was then made through the still damp skin area to the right of the spine, the needle being directed so that the injected cell mass was finally placed intramuscularly into the dorsal aspect of the left thigh or hip. This gave a very long needle track with minimal probability of loss of cells on withdrawal of the syringe, as well as reduced likelihood of infection creeping along the needle track and into the area where the in-

[1] Received for publication December 12, 1949.

[2] Acknowledgment is also made of the technical assistance of Josephine Kahler, Rosslyn Wallace, Grady Carter, Leslie Hall, and Ellison Peppers.

TEXT-FIGURE 1.—Funnel used in loading tissue clump into syringe. The funnel is drawn from 12 mm. O. D. glass tubing.

jected cells lay. Following injection the area of the skin treated with alcohol was coated lightly with melted vaseline to prevent skin sloughing, which sometimes resulted from the action of absolute alcohol on the skin if vaseline was not used.

All mice used for these injections have been strain C_3H, Andervont substrain, and have been obtained from the supply colony of this Institute. Most injections have been made not long after weaning of the mice, but a few have been made in animals about 6 months of age. Data kept with respect to mouse age have not been sufficiently detailed to allow analysis for each culture. Most of the mice used have been males. No differences in the reactions of males and females to the injection was noted. When females were used, owing to shortage of males, particular care has been taken to exclude mammary and lymphoid tumors from the data presented. The limited occurrence of these tumors in such young mice, together with the fact that the injected tissue was placed intramuscularly in the thigh or hip, made it easy to distinguish and discard data from mice with such spontaneous tumors. All such questionable tumors have been confirmed by microscopic examination.

In all, five groups of injections from tissue cultures have been made into C_3H mice. Data from the first of these has already been detailed (*6*). The larger number of tumors that appeared showed up in from 7 to 30 days, and only an occasional tumor appeared after 70 days. In groups 2, 3, 4, and 5 an attempt was therefore made to carry all mice showing no immediate tumors at least 120 days, although a few on which records were carried for only 90 days have been included. Tumors were allowed to reach at least 1 cm. in diameter. Post-mortem examination was done on some tumor-bearing animals, and sections of the tumor were studied microscopically. In other instances the tumor was allowed to grow until

it killed the animal. In some instances where the tumor was allowed to produce death, it was not possible to obtain microscopic sections due to loss of the mouse by cannibalization or by decomposition.

The five groups of injections made from tissue cultures into strain C_3H mice are as follows:

Group 1.—Injection from March 3, 1942, to December 1, 1942. Detailed data from this group have already been presented (*6*) but are included in text-figure 2 in this article for the sake of completeness.

Group 2.—Injected from June 28, 1943, to September 30, 1943.

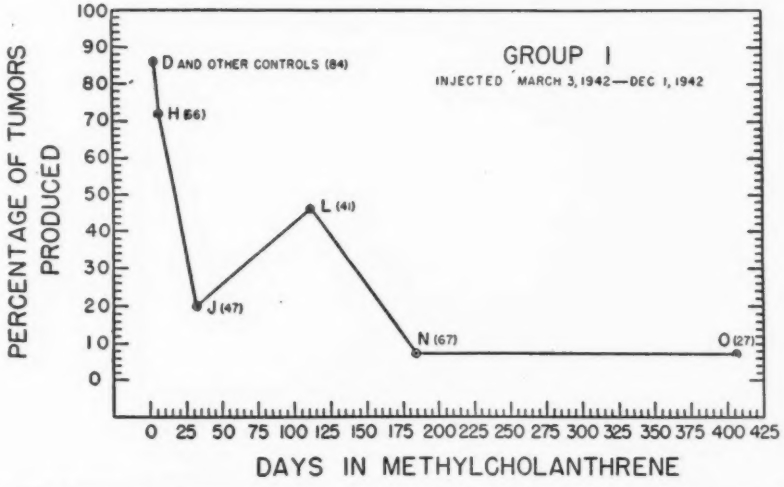
Group 3.—Injected from July 6, 1944, to September 7, 1944.

Group 4.—Injected from December 20, 1944, to February 17, 1945.

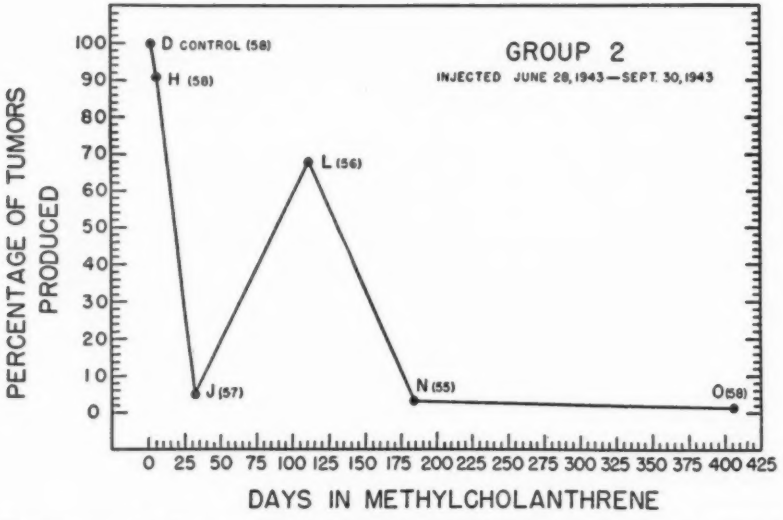
Group 5.—Injected from September 13, 1946, to November 8, 1946. This group of injections was made from the cells of strain D (control) and L only.

RESULTS

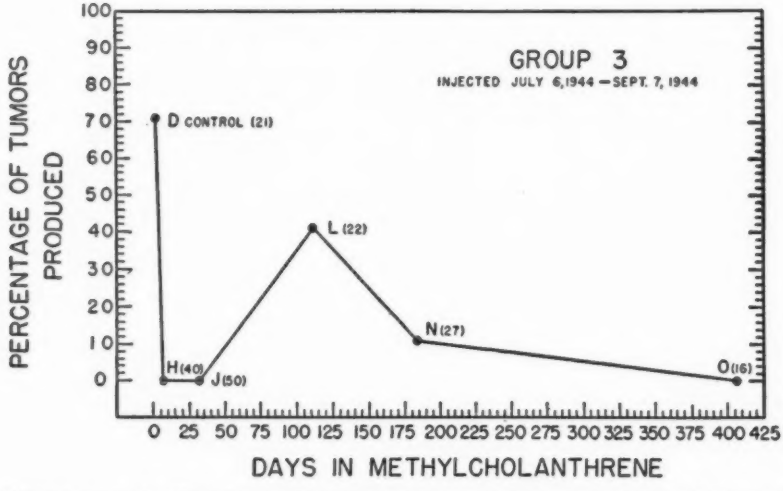
In text-figure 2 of a preceding article (*6*) the percentage of tumors produced by injections of cultures of each cell strain is plotted against the number of days each strain was treated with 20-methylcholanthrene. This figure is reproduced for comparison as text-figure 2 of the present article. The results from later groups of injection, plotted in a similar manner, are presented as text-figures 3, 4, 5, and 6 respectively.



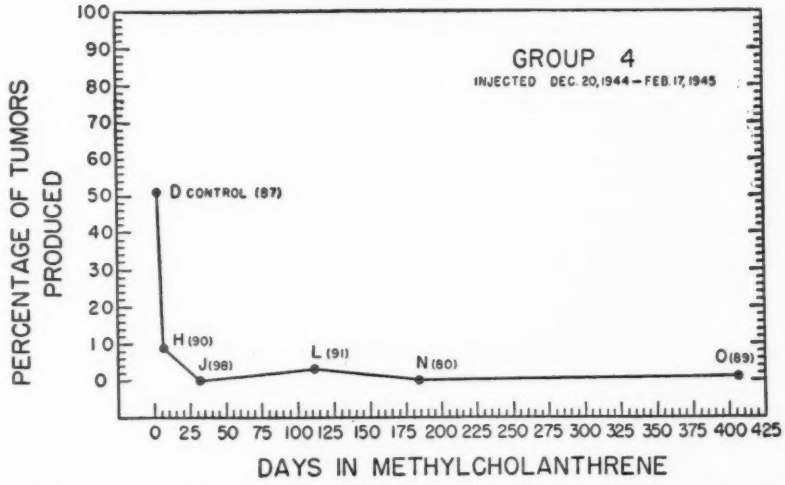
TEXT-FIGURE 2.—Percentage of tumors induced by injection of cultures of Group 1. The abscissa indicates the number of days the individual cell strains studied were treated with 20-methylcholanthrene, while along the ordinate is indicated the percentage of tumors produced for each cell strain. The number beside each point indicates the number of mice injected to determine that point, while the letter identifies the cell strain used. In conformity with the convention used in the earlier presentation (*6*) the strain D (control) point is shown slightly to the right of the zero point. The same method of presentation will be used for text-figures 3, 4, 5, and 6.



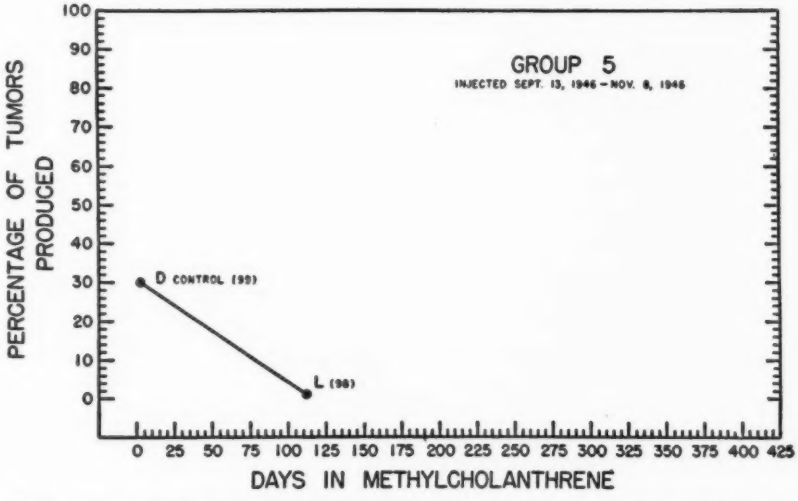
TEXT-FIGURE 3.—Percentage of tumors induced by injection of cultures of Group 2.



TEXT-FIGURE 4.—Percentage of tumors induced by injection of cultures of Group 3.



TEXT-FIGURE 5.—Percentage of tumors produced by injection of cultures of Group 4.



TEXT-FIGURE 6.—Percentage of tumors produced by injection of cultures of Group 5.

DISCUSSION

The injections of strain D cells, during the whole period of study gave the greatest percentage of tumors of any of the cell strains when plotted as in text-figures 2, 3, 4, 5, and 6. From this high value the percentage curve dropped down to the very low value for strain J, rose sharply for strain L, and then dropped to a very low value for strains N and O. The reason for this irregular shape of the curves is not indicated by data so far accumulated, but that this general shape of curve is significant is emphasized by its repetition in data from injections of each of the cell groups studied, and by its repetition from other phases of the data presented. A more detailed consideration of this shape of curve will be postponed until a later date.

The change in the percentage of tumors produced by injections of each of the cell strains of Groups 2 (text-fig. 3) as compared with those of Group 1 (text-fig. 2) appears to be of dubious significance; however, when the general trend of these five curves (text-figs. 2–6) is considered over a period from early 1942 to late 1946 it appears that there has been a general and substantial decrease in the ability of these cell strains to give rise to tumors in C_3H mice. Further, this decrease is either indicated or suggested for each of the six cell strains on which data are available.

Data available do not clearly indicate why with the passage of time each of these cell strains lessened in its ability to produce tumors on reinjection into mice. Several possibilities must be considered. The first of these is that the number of cells on each succeeding culture generation might have become fewer and fewer. However, this was not the case. Each mouse was injected with one culture. The later cultures were fully as luxuriant in their growth *in vitro* as were those of the corresponding cell strain at an earlier date. Consequently there is every assurance that each of the mice of the later series was injected with fully as many vigorous, living cells as was each mouse of the earlier series.

A second possibility is that the strain of mice used for injection may have undergone some genetic alteration that lessened the possibilities of obtaining tumors. However, of numerous studies carried on in this Institute on this strain of mice, none have suggested any such mouse-strain alteration. Further, the ability of the cell injections to induce tumors appeared to lessen slowly, and a genetic change in the mice would more probably have been indicated by a sudden change in the incidence of tumors. Finally, tumor strains originated from cell strains H, L, N, and O by injection into this same C_3H strain of mice were successfully carried through more than 60 generations of subinoculation with essentially 100 percent takes [3] and without tendency to regression. This uniformity of behavior, while not conclusive, would also indicate that with respect to these cell strains the mouse strain had been essentially stable from the time the cells of these four strains were first reinoculated into it in 1942.

[3] More detail on these later generations of *in vivo* transplantations will be given in a later paper.

A third possible explanation is that within the period of time of these injection studies some cell type specifically capable of giving rise to tumors on injection was gradually eliminated from the cultures. If this had occurred in any cell strain the ability of that cell strain to give rise to tumors might gradually have lessened. However, if this did occur it occurred in each of the six cell strains. If it did occur it was without any recognizable change in the morphology of the cells in strains D, H, and L. This explanation appears to be unlikely.

A fourth possibility to be considered is that the period of accidental overheating of the cell strains in tissue culture in May 1944 (*2*) may have altered the cells in such a way as to progressively lessen their ability to produce tumors on reinjection. The period of overheating preceded by 4 months the third group of injections (text-fig. 4) in which the first major decline in tumor percentage was recorded. For this reason the possibility that the heat had some effect upon the cultured cells cannot be disregarded. The data indicate that the heat effect would have had to be of such a nature that the cells slowly but progressively lost their ability to produce tumors when injected into mice. Present knowledge does not permit a final evaluation of the effect of heat on cells, but it is reasonable to assume that such an effect would be similar to a genetic alteration where the change would be abrupt rather than cumulative and gradual. Furthermore, a similar loss of malignancy in cultured cells was observed by DeBruyn (*8*) who, in recent work with mouse lymphosarcoma cell strain MB 13, reported that when these cells were grown in an entirely heterologous medium for more than 4 months, they were no longer able to give rise to tumors upon reinjection into mice. Her cultures were presumably never overheated. It would seem, therefore, that the loss of malignancy of the cultures in this laboratory cannot be explained by the 2-week period of irregular overheating to which they were subjected.

A further possibility to be considered is that the changes induced in these six cell strains are subsiding or are undergoing modification so that the cells less easily give rise to sarcomas on injection into mice. It may be that the original alteration that allowed the cells to give rise to sarcomas was a temporary one and that the capacity of the cells to produce tumors was destined to decrease with the passage of time. However, tumors originated from the cells of strains H, J, L, N, and O have been carried through more than 60 generations of animal transfer [4] without any evidence to indicate lessening of the ability to grow sarcomas on subinoculation. Since the lessening did not occur *in vivo*, it seems unlikely that the decrease in tumor production was the manifestation of an intrinsic mechanism of the cell.

It appears more likely that the cultivation of the cells for over 5 years, entirely outside of their original host and in a completely heterologous culture medium made up of horse serum and chick embryo juice, has induced changes in the cells that have lessened their ability to give rise to sarcomas on reinjection into C_3H mice. These changes may well reflect some loss

[4] Detailed information on subinoculation of the sarcoma strains will be given in a later paper.

of the species specific reaction or some other alteration of the reaction of the cells to the host.

A similar lessening of the ability of cultured cells to give rise to sarcoma on reinjection *in vivo* was observed by DeBruyn (*8*) with mouse lymphosarcoma cells. In this instance, too, an entirely heterologous medium was used.

Whatever the cause may be, until the reason for this decreased ability of each of the cell strains to give rise to sarcomas on reinjection is explained and brought under experimental control, a serious question is raised regarding the significance and validity of interpretations of negative results from reinjection of any strain of cultured cells into animals. Negative results from injection of any such suspected cells would appear to be of extremely equivocal and uncertain significance.

In the case of this group of cell strains, it appears that the progressive cumulative action of a chemically defined transforming agent ultimately produced cell changes so severe that in the extreme cases of strains N and O, the cells became less thrifty in their growth in culture and less able to readjust themselves when returned to life *in vivo*. Even in 1942 when injected cultures of strains D and L were giving 88 and 47 percent of tumors respectively, strains N and O were giving less than 10 percent. This low percent dropped still lower with the passage of time in culture. Study of the culture line genealogy of cultures of strains N and O, which were able to induce sarcomas, showed no segregation of a more powerful sarcoma-inducing line of cells or cultures within either of these two strains. It appeared, rather, that the tumor-producing ability of the cells of these two strains as a whole had greatly decreased.

The cells of these N and O strains in culture also showed the most extreme morphologic alterations of any of the cells as compared with the normal fibroblasts from which they arose (*1*). Although cells of these two strains gave rise to sarcomas on injection in only occasional instances, the cells of the sarcomas produced showed extreme morphologic anaplasia (*7*). These data indicate apparently unequivocally that in such cell systems, in which all cultures originally arose from a single parent culture, the degree of morphologic anaplasia of the cells cannot be considered as directly correlated with the ability of the cells to give rise to sarcomas on injection into animals of the host strain. To the contrary, it appears that cells may be so greatly altered morphologically and physiologically by the action of a transforming agent, such as 20-methylcholanthrene, that they become less thrifty in culture and less able to readjust themselves to life *in vivo*. The mechanism and nature of these changes must await further elucidation.

It appears quite probable that similar extreme cell changes under the action of a transforming agent might occur *in vivo*. For instance, a transforming agent may progressively alter a cell strain to where its cells demonstrate increased autonomy of proliferation *in vivo* so that a tumor results. With continued action of the carcinogen on the cells, a condition of maximum autonomy must result sooner or later, and, from this stage on, further progressive alterations of the cells by the same agent

may so progressively handicap the cells of the strain that ultimately they are no longer able to exist, and so must die. The study of physiologic changes involved in such progressive alteration would appear to be most interesting and significant in cancer research.

SUMMARY AND CONCLUSIONS

1. In order to test the ability of six tissue culture strains of altered mouse fibroblasts, previously described, and designated as strains D (control), H, J, L, N, and O, to give rise to tumors on injections, cultures of each were injected into C_3H strain mice. Five groups of injections were made between March 3, 1942, and November 8, 1946. In each group of injections some cultures of each cell strain usually gave rise to sarcomas.

2. The percentage of sarcomas produced in the first group of injections was highest for strain D, lower for strain H, down to very low for strain J, rising to a second peak for strain L, and dropping to an extreme low for strains N and O.

3. This general relationship was constant for the cell strains within each group of injections. In groups 3, 4, and 5, however, the general level of the whole curve was gradually lowering with the passage of time.

4. Reasons for this lowering are considered. The most probable concept is that the growth of the cultures in an entirely heterologous culture media for such an extended period of years has gradually altered the characteristics of the cells to a point where they can less easily live and give rise to sarcomas when injected.

The data indicate that in cell systems such as this one that has been studied, where all cultures arose from one parent culture, the degree of morphologic alteration of the cells cannot be considered as directly correlated with the ability of the cells to give rise to sarcoma on injection into animals of the same strain. To the contrary, it appears that cells may be so greatly altered morphologically and physiologically by the action of a transforming agent such as 20-methylcholanthrene that they are less thrifty in culture and less able to readjust themselves to life *in vivo*.

REFERENCES

(*1*) EARLE, W. R.: Production of malignancy in vitro; mouse fibroblast cultures and changes seen in living cells. J. Nat. Cancer Inst. 4: 165–212 (1943).

(*2*) EARLE, W. R., SCHILLING, E. L., and SHELTON, E.: Production of malignancy in vitro; description of cells at the fluid interface of the culture. J. Nat. Cancer Inst. 10: 865–882 (1950).

(*3*) ———: Production of malignancy in vitro; continued description of cells at the glass interface of the cultures. J. Nat. Cancer Inst. 10: 1067–1101 (1950).

(*4*) EVANS, V. J., and EARLE, W. R.: The use of perforated cellophane for the growth of cells in tissue culture. J. Nat. Cancer Inst. 8: 103–119 (1947).

(*5*) SANFORD, K. K., EARLE, W. R., and LIKELY, G. D.: The growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst. 9: 229–246 (1948).

(*6*) EARLE, W. R. and NETTLESHIP, A.: Production of malignancy in vitro; results of injections of cultures into mice. J. Nat. Cancer Inst. 4: 213–227 (1943).

(*7*) NETTLESHIP, A., EARLE, W. R.: Production of malignancy in vitro; pathology of tumors produced. J. Nat. Cancer Inst. 4: 229–248 (1943).

(*8*) DeBRUYN, W. M.: A study in vitro of a strain of lymphoblast-like cells, MB13, derived from mouse lymphosarcoma MB (T86157). Anat. Rec. Vol. 103 page 22, March (1949).

A COMPARATIVE STUDY OF THE MORPHOLOGY AND GLUCURONIDASE ACTIVITY IN 44 GASTROINTESTINAL NEOPLASMS [1]

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The presence of β-glucuronidase activity in increased amounts was noted in human cancer tissue by Fishman and coworkers (*1*). A wide variety of primary neoplasms and of secondary metastatic lesions was studied, and it was felt that in the majority of cases an elevated glucuronidase activity may constitute a characteristic of cancer tissue.

The suggested functions of β-glucuronidase have been (a) metabolic conjugation of steroid hormones (estrogens) (*2*), (b) *in vivo* hydrolysis of conjugated glucuronides (*3*), and (c) cell proliferation (*3*). It is of considerable interest to determine whether the function of the enzyme in cancer tissue is the same as, or different from, the ones suggested hitherto.

It is important also to evaluate more exactly the pathologic significance of the presence of increased glucuronidase in cancer tissue. Is it related to nonmalignant processes such as inflammation, necrosis, fibrosis, and mucin formation or to the properties of malignant disease, which include the state of cellularity, differentiation, mitotic activity, and the degree of penetration of surrounding tissues by the lesion and involvement of lymph nodes?

With a view to obtaining preliminary information that would be relevant to the function and pathologic significance of glucuronidase in cancer tissue, a comparative study has been made of the morphologic appearance and the glucuronidase activity of tissues taken from gastrointestinal neoplasms.

METHODS AND PLAN OF STUDY

Essentially the same methods of sampling and assaying β-glucuronidase activity of human tissues removed surgically were employed as previously described (*4*).

In classifying the morphologic variables of these tumors an arbitrary scale of 1+ to 4+ has been used. Frequently these characteristics differ considerably in various parts of the same tumor.

[1] Received for publication December 10, 1949.

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The following criteria were applied to each tumor:

Degree of penetration: 1+ indicates tumor confined to the mucosa, 2+ tumor into the muscularis, 3+ tumor through the serosa, and 4+ any tumor invading beyond the bowel wall or with lymph-node metastases. *Degree of differentiation:* 4+ represents those tumors that form the most perfect glands or most mature pearls, *etc.*, whereas tumors listed 1+ are the least differentiated. *Relative cellularity:* an estimate of the number of tumor cells per high-power field, 4+ indicating the most cellular tumors. *Mitoses:* an indication of the malignancy and speed of growth, based upon estimates of the number of mitoses per high-power field, 4+ representing the highest number. *Inflammation:* many tumors are ulcerated and the stroma beneath the ulcers contains many acute and chronic inflammatory cells; the tumors with most infection are classed 4+. In Case 30, there is an unusual lymphatic response surrounding the tumor, apparently a defense reaction of the individual to the neoplasm, which is benign in many characteristics. *Necrosis:* in many tumors there is considerable necrosis of stroma and tumor cells, probably indicating that the tumors have outgrown their blood supply. The greatest degree of necrosis is classed 4+. In many tumors the necrosis seems to be confined to large tumor glands. *Fibrous stroma:* an estimate of amount of fibrous stroma per high-power field, 4+ indicating the less cellular and consequently more fibrous tumors. *Mucin:* occasional gastrointestinal adenocarcinomas form large quantities of mucin; those with the greatest amounts are classed 4+.

In table 1 are listed the data obtained in the gastrointestinal series, in table 2 lymph nodes, and in table 3 gastrointestinal polyps.

TABLE 1.—*The relationship of the glucuronidase activity of gastrointestinal tumors to their morphologic appearance*

Patient (identification No.)	Surgical pathology unit No.	Glucuronidase activity		Morphologic appearance								
		Uninvolved	Tumor	Number of lymph nodes involved	Local penetration	Degree of differentiation	Relative cellularity	Mitosis	Inflammation	Necrosis	Fibrous stroma	Mucin
CARCINOMA OF THE ESOPHAGUS AND CARDIA												
2	28718	750	1570	0/5	2+	2 to 3+	3+	2+	3 to 4+	4+	1+	1+
3	28757	647 (esophagus) 1270 (stomach)	2160	4/4	4+	1+	4+	2+	1+	1+	1+	1+
10	29079	486 (esophagus) 720 (stomach)	853	0/11	2+	3+	3 to 4+	3+	3 to 4+	2+	1 to 2+	1+
15	29625	1376 (esophagus) 724 (stomach)	2753	2/10	3+	3+	3+	2+	3 to 4+	4+	1+	1+
29	30465	1077 (esophagus)	1725 (center)	12/16	4+	4+	3+	2+	4+	1+	2+	4+
			1107 (edge)	----------	4+	4+	3+	2+	4+	1+	2+	4+
39	31247	144 (mucosa) 123 (muscle)	1300	2/7	1+	1+	4+	1 to 2+	1+	1+	1+	1+
CARCINOMA OF THE STOMACH												
5	28826	940	3180	4/9	4+	1+	3 to 4+	2+	1 to 3+	4+	1+	3 to 4+
9	28965	437	1538	21/21	4+	1+	1 to 2+	2+	1+	1+	4+	1+
11	29252	191	1640	0/17	3+	1 to 2+	4+	2+	3+	4+	1+	2+
12	29314	636	2412	2/9	4+	1+	4+	3+	2+	2+	1+	1+
18	29748	269	2810	0/1	3+	1+	4+	2 to 3+	2 to 3+	1+	1+	1+
19	29977	----------	811	4/4	4+	1+	2+	1+	1+	1+	3+	2+
20	29863	1958	3575	7/9	4+	3 to 4+	3+	2+	3+	1 to 2+	1 to 2+	2+

TABLE 1.—*The relationship of the glucuronidase activity of gastrointestinal tumors to their morphologic appearance*—Continued

CARCINOMA OF THE STOMACH—Continued

Patient (identification No.)	Surgical pathology unit No.	Glucuronidase activity		Morphologic appearance								
		Uninvolved	Tumor	Number of lymph nodes involved	Local penetration	Degree of differentiation	Relative cellularity	Mitosis	Inflammation	Necrosis	Fibrous stroma	Mucin
28	30455	449	905 (edge)	10/11	4+	2 to 3+	3+	2+	2+	4+ (surface) 1+ (deep)	2 to 3+	1+
		607	406 (center)	----------	4+	3+	3+	2 to 3+	2 to 4+	2+ (in glands)	2+	2 to 3+
35	30962	457	2640	----------	4+	1 to 2+	3 to 4+	1+	2 to 4+	1+	1 to 2+	3+
43	31344	520 (1 cm)*	549 (edge)	9/12	4+	4+	2 to 3+	2 to 3+	3+	2+ (in glands)	2 to 3+	1+
		430 (5 cm)	489 (center)	----------	4+	3 to 4+	2 to 3+	2 to 3+	3+	4+ (on surface)	3+	1+

*Approximate distance of assayed specimen from edge of tumor.

CARCINOMA OF THE COLON AND RECTUM

1	28601	1930	900	1/9	4+	4+	2+	1+	2 to 3+	2+ (surface)	3+	1+
4	28803	1760	1360	0/1	4+	4+	2+	1 to 2+	1+	1+	2+	3 to 4+
6	28828	2490	5250	4/10	4+	4+	2 to 3+	2+	2 to 4+	1+	2 to 3+	1+
7	28881	1524	2890	0/8	4+	4+	2 to 3+	2+	1 to 2+	3 to 4+	1 to 2+	1+
8	28912	1480	1320	0/4	4+	4+	2+	1+	2+	1+	3+	4+
13	29480	364	2400	5/8	4+	4+	3+	2+	2+	2 to 3+	2+	1+
14	28995	449	827	16/17	4+	4+	2+	2 to 3+	3+	3+ (surface)	3+	4+
16	29632	2635	2185	----------	3+	4+	2+	2+	2 to 3+	1+	2+	1+
17	29706	----------	700 A	7/17	4+	4+	2+	2+	4+	2+	3+	1+
			17,300 B	7/17	1+	4+	3+	2+	2+	1+	1+	2+
			6800 C	7/17	4+	4+	2+	1+	1+	1+	2 to 3+	1+
21	29881	----------	2107	----------	----------	3 to 4+	3+	2 to 3+	2+	4+	1+	1+
22	30251	2782 (1 cm)	1859	0/4	4+	4+	3+	3 to 4+	2 to 3+	1+	2+	3+
		1368	2423	----------	4+	4+	2+	2 to 3+	1+	2+	2 to 3+	2+
23	30267	1409 (1 cm)	1143 (infilt)	----------	4+	4+	2+	2+	1+	2 to 3+	3+	1+
		1569	1501	----------	4+	4+	2 to 3+	2+	1+	1+	3+	1+
24	29758	1310	2810	0/1	4+	4+	3+	2 to 3+	2 to 3+	1+	2+	2 to 3+
25	30324	1673 (1 cm)	3787 (edge)	----------	4+	4+	3+	2 to 3+	2+ (in glands)	2 to 3+	2+	1+
		934 (2.5 cm)	3862	----------	4+	4+	2+	2 to 3+	1+	2+	3 to 4+	1+

		934 (2.5 cm)	3862 (center)	----------	4+	4+	2+	2 to 3+	1+	2+	3 to 4+	1+
26	30345	1253 (1 cm)	4150 (edge)	0	4+	4+	3+	3+	2 to 3+	2+	2+	1+
		1056 (10 cm)	1555 (center)	----------	4+	4+	2+	2+	2+	2+	3+	1+
27	30387	4240 (½ cm)	2960 (edge)	----------	1+	1+	4+	2 to 3+	3 to 4+	1+ (surface)	1+	1+
		4440 (1 cm)	2157 (center)	----------	1+	1+	3 to 4+	2 to 3+	2 to 3+	1+	1+	1+
		2065 (15 cm)										
30	30438	742 (1 cm)	1733 (edge)	0/14	3+	4+	3+	3 to 4+	2 to 3+	2+	1 to 2+	1+
		474 (4.5 cm)	2196 (center)	----------	3+	4+	3+	4+	2+ (with 4+ lymphocytic response)	1+	1+	2+
31	30538	531 (1 cm)	2280 (edge)	2,2	2+	4+	3+	2+	3+	1+	1 to 2+	1+
		1143 (4 cm)										
32	30554	1414 (1 cm)	6110 (white material)	----------	1+	----------	1+	----------	2+	0+	0+	4+
		1110	5965	----------	1+	4+	4+	2 to 3+	1 to 2+	1+	1+	1+
33	30657	543 (1 cm)	1202	0/10	4+	4+	3+	2+	1 to 2+	1+	2+	4+
34	30874	1700 (1 cm)	4000 (edge)	----------	3+	4+	3 to 4+	1 to 2+	2+	+	1 to 2+	1+
		1450 (15 cm)	2300 (center)	----------	3+	4+	2 to 3+	1 to 2+	2 to 3+	3+ (on surface)	3+	1+
36	31045	669 (1 cm)	1474 (edge)	0/15	4+	3+	2 to 3+	2 to 3+	3+	3+	2+	1+
		442 (5 cm)										
37	31084	702 (7 cm)	3140 (edge)	1/14	4+	4+	3+	2+	1+	1 to 2+	2+	1+
38	31182	370 (1 cm)	1123 (edge)	2/7	4+	4+	3+	2+	2+	2+ (in glands)	2+	1+
		454 (7 cm)										
40	31274	690	820	0/2	2 to 3+	4+	3 to 4+	3+	3+	2+	2+	1 to 2+
41	3034	1651 (1 cm)	3510 (edge)	3/6	4+	3 to 4+	2+	1+	2 to 3+	1+	3+	1 to 2+
		1394 (far)	1251 (center)	----------	4+	4+	1 to 2+	1+	1+	3+ (on surface)	3+	2 to 3+
42	30809	503 (near)	3262 (edge)	0/1	4+	4+	2 to 3+	2 to 3+	3+	2+ (on surface)	2+	2+
		1550 (far)										
44	31365	930 (1 cm)	1570 (edge)	0/7	3+	4+	3+	2 to 3+	4+	2+	2+	1+
		1560 (15 cm)	1550 (center)	----------	3+	4+	2 to 3+	1 to 2+	2 to 3+	2+	2 to 3+	1+

TABLE 2.—*Glucuronidase activity of lymph nodes*

Patient (identification No.)	Involved organ	Lymph node assays for glucuronidase activity (units)	
		Uninvolved	Involved
9	Stomach	104	1522, 1565, 1455, 1533, 1457, 1085, 1660
10	do	1590	
13	Colon	1213	1550
45	Stomach		7220
46	Colon		2912
22	do	160	
23	do	425	
41	do	480, 450	
27	do	2000, 750	
28	Stomach		1080
29	Esophagus		1244, 1914
30	Colon	2908, 2150	
31	do		1236
36	do	1765	
37	do	336	
39	Esophagus		1020

TABLE 3.—*Glucuronidase activity of gastrointestinal polyps*

Patient (identification No.)	Diagnosis	Glucuronidase activity	Remarks
47	Ca of rectum	50	Extremely benign inflamm. 2+, stroma +, mucin 2+, cell. 2 to 3+, no hyperchromaticity, no mitoses.
33	Ca of sigmoid	1274	Inflamm. 2+, stroma 1+, cell. 3 to 4+, mucin 2+, penet. 1+, mitosis 1 to 2+. Undergoing change toward malignancy.
34	Ca of colon	4500	Inflamm. 1+, stroma 1+, cell. 3 to 4+, mucin 3+, penet. 1+, mitosis 1+.
41	Ca of rectum	(1) large 3747	Inflamm. 2+, stroma 1+, cell. 2+, mucin 3+, mitosis, hyperchromaticity, no tumor, benign.
		(2) small 3840	Inflamm. 2 to 3+, stroma 1+, cell. 3+, no invasion, some mitosis, no tumor, benign.

RESULTS

When the data are grouped according to + class and average it is clear that one cannot point to a single morphologic characteristic studied that alone accounts for the amount of glucuronidase found in the various tissues. Assuming that the number of samples studied would be representative of a much larger series of observations, one can probably exclude the nonmalignant characteristics as being important factors in explaining glucuronidase activity, *e. g.*, inflammation, necrosis, formation of fibrous stroma, and mucin.

Of the malignant characteristics, the lymph-node involvement, degree of penetration, and differentiation appear to bear little relation to the results.

As the amount of necrosis and fibrosis increases, the activity of glucuronidase per gram of tissue decreases to a slight extent. There is an increase in glucuronidase associated with the greater cellularity of the lesion (*e. g.*, ++, average 1960; +++, 2240; ++++, 2950). In judging mitotic activity (+, 1670; ++, 2580; +++, 2590), an effect may have been present in going from 1 to 2+ but not afterwards.

With regard to lymph nodes (table 2) and gastrointestinal polyps (table 3), a number were found in which the elevated glucuronidase was not accompanied by morphologic evidence of malignant disease. Occasional sections of apparently uninvolved gastrointestinal mucosa sometimes gave high titers.

DISCUSSION

The choice of gastrointestinal cancer as the type of malignancy for this study was made because gastrointestinal adenocarcinoma tissue is regarded as being homologous with gastrointestinal mucosa. In this way, comparisons of any one characteristic of tissues, uninvolved and involved with cancer, have more validity than in cases where the tissues of comparison are not homologous.

The limitations of the methods employed here should be noted. Thus, one to three sections of each tumor were examined and from these a decision was reached with regard to the morphologic characteristics of the entire tumor. It is possible that the sections viewed were not representative. It is common knowledge that the morphology of one tumor may vary extensively from one microscopic field to another. Moreover, it is assumed that the tissue homogenized for enzyme assay possessed a histologic picture identical to the adjacent area of the tumor taken for microscopic study. Accordingly, it was to be expected that some tissues with elevated glucuronidase would not show tumor in the adjacent areas.

Of interest is the occurrence of rather high glucuronidase values in polyps that are not malignant but that exhibited characteristics suggesting precancerous changes. The finding of high glucuronidase in some lymph nodes that were not involved (to a noticeable extent) with cancer may possibly be explained by the collection in the lymph nodes of products such as the enzyme glucuronidase emanating from the primary tumor.

In general, the present results confirm our previous findings in which enhanced tumor glucuronidase activity was found in the majority of cases of human cancer. In addition, they indicate that occasionally uninvolved intestine, lymph nodes, and polyps may exhibit increased activities that may possibly be explained as characteristic of a precançerous state. The increased glucuronidase was not clearly related to any one of the morphological characteristics examined. This does not exclude the possibility that it may be. It is believed that this question will be settled by the application in the future of histochemical or other techniques. The pos-

sibility exists that glucuronidase activity may not be accompanied by any specific morphologic manifestation, in which case even most refined methods would not demonstrate a correlation between the histologic picture and enzyme activity.

SUMMARY

A study was made of the β-glucuronidase activity of 44 malignant neoplasms of the gastrointestinal tract. Of these, 37 showed a greater glucuronidase activity in the tumor as compared with the activity found in the uninvolved homologous tissues of origin. It was not possible to demonstrate a correlation of these enzyme values with the morphologic features examined, *i. e.*, local penetration, degree of differentiation, relative cellularity, mitosis, inflammation, necrosis, fibrous stroma, and mucin. In a few instances in which no histologic evidence of tumor existed, high values were found in tissues adjacent to the tumor, in lymph nodes, and in polyps.

REFERENCES

(*1*) Fishman, W. H., Anlyan, A. J., and Gordon, E.: β-Glucuronidase in human tissues: some correlations with processes of malignant growth and with physiology of reproduction. Cancer Research 7:808–817 (1947).

(*2*) Fishman, W. H.: β-glucuronidase; its relation to action of estrogenic hormones. J. Biol. Chem. 169: 7–15 (1947).

(*3*) Levvy, G. A., Kerr, L. M. H., and Campbell, J. G.: β-glucuronidase and cell proliferation. Biochem. J. 42: 462–468 (1948).

(*4*) Fishman, W. H., Springer, B., and Brunetti, R. J.: Application of improved glucuronidase assay method to study of human blood β-glucuronidase. J. Biol. Chem. 173: 449–456 (1948).

INHIBITION OF SEA-URCHIN EGG CLEAVAGE BY A SERIES OF SUBSTITUTED CARBAMATES [1]

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BACKGROUND

The remarkable properties which urethan has recently been found to possess endow it with a compelling interest for the experimental oncologist and cellular physiologist. Only in 1943 (*1*) was it discovered that, in addition to narcosis, urethan could induce pulmonary tumors in mice. This has been substantiated by later reports of tumor induction in mice (*2–6*) and rats (*7*, *8*). Then in 1946 it was announced that urethan inhibits the growth of animal tumors (*9*) and is of value in treating human leukemia (*10*). An extensive sampling of the clinical, cytological and enzymological work with urethan has been reviewed by Heilmeyer, Merk, and Pirwitz (*11*).

Urethan thus proves to be another carcinogen we can add to the list of tumor-inhibitors. This paradoxical property of carcinogens has been recently surveyed by Boyland (*16*). Haddow and his coworkers reported 10 years earlier their discovery of the retarding effect that carcinogenic hydrocarbons exert upon normal and malignant growth (*17–19*). Recently a number of studies of this interference have been reported (*22–29*). It is pertinent to note that the above studies and others (*20*, *21*) also reveal an inhibition of normal growth, and there is retardation of normal regeneration, in the form of wound healing, by tar (*30*).

As an attempt at isolating the process upon which the carcinogens act when they inhibit growth, it was decided to compare the effectiveness of a series of simple carbamates as to their efficiency in stopping the cleavage of marine eggs. The argument is simply this: if the inhibition of cell division is an essential step in carcinogenesis, we shall obtain correlation between inhibition of mitosis here and the induction of lung tumors as reported by others. Simultaneously, we are interested in the effect of chemical constitution upon the effectiveness of a series of closely related

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[3] This work was supported by a grant from the American Cancer Society, Inc. to the Sloan-Kettering Institute.

compounds, both for what it may reveal of the mechanism of mitosis, and as a guide for selection of substances for chemotherapy of cancer.

An abundance of literature on the effects of carbamates on marine eggs and other dividing cells dates from Warburg's early studies of narcosis (*31*). These will be discussed in a later paper.

MATERIALS AND METHOD

Eggs

The eggs of two subtropical sea urchins were used: *Tripneustes esculentus* and *Lytechinus variegatus*. The first offers the advantage of a large number of eggs for sectioning. The second has an egg of extraordinary transparency, permitting observation of changes in the aster, spindle, and nucleus within the living cell.

The eggs and sperm were obtained by inducing shedding or by removal of the gonads. The eggs were usually strained through gauze and then through bolting cloth to remove debris and jelly, and were given three or four washings in ten times their volume of sea water. A few hundred eggs were then added to 10 ml. of experimental solution or control sea water.

Exposure was continuous, beginning 9 to 11 minutes after fertilization. At this time the sperm head is swelling and forming an aster, but the pronuclei have not fused, so that the mitotic cycle has not yet begun. Cleavage occurs 90–100 minutes after fertilization at 23.0°±0.1° C, or at 50–60 minutes at 25.75°±1.0° C in *Tripneustes*, and at 57–62 minutes at 23°± 0.1° C, or 45–50 minutes at 25.75°±1.0° C in *Lytechinus*. Most experiments were run at 23°. The exceptional 26° tests are indicated.

Compounds[4]

The hypothetical carbamic acid H_2NCOOH serves as the basis for a series of esters, with, in some instances, one or two substitutions on the amino group. The single alkyl groups of the esters listed in table 1 extend from methyl to dodecyl. In table 2, one (e. g., *methyl*) or both (e. g., *dimethyl*) hydrogens of the amino nitrogen are replaced by alkyl or aryl groups, of successively greater weight. Two urethan molecules can be joined at the nitrogen atom directly (diurethan) or by some connecting group ($C_2H_5OOCNHR'NHCOOC_2H_5$). Those included in table 3 are joined by alkyl chains containing one to six carbons or by different aromatic groups. The other symmetrical molecule pattern joins two or three substituted carbamic acids by the oxygen atom. These *bis-* and *tris-*carbamates esterified on a single phenyl ring (R′R″ NCOO–ϕ–OOCNR′ R″) are in table 4. Since the molecules are symmetrical, one column

[4] I am indebted to Dr. Howard E. Skipper for a supply of methyl carbamate, and to Dr. C. D. Larsen for 24 of the carbamates. Dr. C. Chester Stock procured all the other carbamates, which came from the following sources: The Abbott Research Laboratories, North Chicago, Ill. (A); The Toxicity Laboratory, The University of Chicago (B); Monsanto Chemical Co., St. Louis, Mo. (C); Merck & Co., Rahway, N. J. (D); Calco Chemical Division, American Cyanamid Co., Bound Brook, N. J. (E); Chemical-Biological Coordination Center, National Research Council, Washington, D. C. (F); Southern Research Institute, Birmingham 5, Ala. (G); Dr. Francis E. Ray, University of Florida, Gainesville, Fla. (H); Goodrich Chemical Co. (J). The letters in parentheses indicate the sources of compounds listed in tables 1–5.

suffices to indicate the single or double substitution on the amino end (R′, R″). Carbamates with sulfur substituted for one or both of the carboxyl oxygens comprise table 5.

The compounds were dissolved directly in sea water when possible. Those with low solubility were dissolved first in ethanol. The eggs were not exposed to concentrations of alcohol higher than 1 percent, which by itself had no effect. Synergistic influences may have been acting at this level, however.

Criteria of Effects

To evaluate the effects of carbamates, the scale used earlier for podophyllin (*32*) has been somewhat modified. Where inhibition was so severe as to prevent division of 95 percent of the eggs, or where there was mere desultory furrowing or when, as is often the case with carbamates, the nucleus divided but no cleavage followed, the eggs were considered incompletely blocked. Where all development quickly ceased upon addition of the drug, the block was graded as complete. Retardation was measured by making a count of 50 eggs 4 to 10 times during the first cleavage and again during the second. The points were connected by straight lines to determine the approximate time of 50 percent cleavage. The eggs were followed until they formed plutei, and a record was kept of the time at which they became blastulae and gastrulae. In the tables these grades of effects are assembled under three major headings: *Block* (complete or incomplete), *First Cleavage Retarded* (time between fertilization and control 50 percent cleavage prolonged by *100 percent* or more, by *10 to 99 percent*, or by *less than 10 percent*), and *Delayed Effect* (second cleavage retarded or embryo retarded). Comparisons within each experiment are based upon the behavior of control eggs from the same female.

RESULTS

The Ester Series

Effectiveness of the carbamate increased as the number of carbons in the ester group increased, up to decyl carbamate. If we compare 100 percent retardation in table 1, the progressive decrease in dose is obvious. The *n*-butyl ester does not appear in this column for the reason that the highest dose tested produced only a 50 percent delay. Nevertheless, this is clearly intermediate between the *n*-propyl and *iso*amyl esters. The *n*-decyl carbamate retarded cleavage 7 percent at 0.55 mM and 0.05 mM as against 0.76 mM *iso*amyl carbamate which was without influence on the first cleavage.

The 12-carbon ester was less effective than those of slightly lower molecular weight, exerting no influence on development at saturation (0.44 mM).

Configuration of the carbon chain affects potency. *Iso*propyl carbamate caused only a slight delay at 19.40 mM and had no effect at 9.70 mM, but it nevertheless is more active than ethyl carbamate. Cyclohexyl carbamate occupies its rightful position as a 6-carbon ester. No straight-chain hexyl carbamate was available.

TABLE 1.—*Esters of carbamic acid—H_2NCOOR*

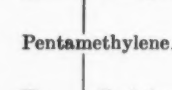
LYTECHINUS

R	Formula	Source	C°	Millimolarity required to induce—						
				Blocking first cleavage complete	Retardation					No effect
					First cleavage			Second cleavage	Embryo	
					100%	10-99%	<10%			
Methyl	$H_2NCOOCH_3$	S	26	-------	106. 64	-------	-------	-------	53. 32	26. 66
Ethyl	$H_2NCOOCH_2CH_3$	D	26	-------	89. 76	44. 88	22. 44	-------	-------	11. 22
iso-propyl	$H_2NCOOCH(CH_3)_2$	L	[1] 23	97. 00	-------	-------	19. 40 9. 70	-------	-------	-------
n-propyl	$H_2NCOO(CH_2)_2CH_3$	L	[1] 23	97. 00	19. 40	-------	9. 70	-------	-------	-------
n-butyl	$H_2NCOO(CH_2)_3CH_3$	L	23	-------	-------	8. 54	-------	-------	-------	-------
iso-amyl	$H_2NCOO(CH_2)_2CH)CH_3)_2$	L	23	-------	7. 62	-------	. 76	-------	-------	. 08
cyclohexyl	$H_2NCOOC_6H_{11}$	E	26	-------	6. 98	-------	1. 40	-------	. 14	. 01
n-decyl	$H_2NCOO(CH_2)_9CH_3$	E, F	26	-------	-------	-------	[2] . 50 . 05	-------	-------	. 005
n-dodecyl	$H_2NCOO(CH_2)_{11}CH_3$	E	23	-------	-------	-------	-------	-------	-------	[3] . 44

[1] Simultaneous tests.
[2] 0.75 percent ethanol.
[3] 1 percent ethanol.

TABLE 2.—*N-substitutions on Urethan: R'R''NCOOCH$_2$CH$_3$*

LYTECHINUS *USED FOR ALL EXCEPT* ISO*PROPYL*

R''	R'	Formula	Source	C°	Millimolarity required to induce—							
					Blocking		Retardation					
					First cleavage		First cleavage			Second cleavage	Embryo	No effect
					Complete	Incomplete	100%	10–99%	<10%			
H	H	$H_2NCOOCH_2CH_3$	D	[1] 23	56. 10		22. 44				11. 22	
H	Methyl	$CH_3NHCOOC_2H_5$	L	[1] 23	49. 95			19. 98 9. 99			1. 00	0. 10
Dimethyl		$(CH_3)_2NCOOC_2H_5$	L	[1] 23		42. 50				17. 00		8. 50
H	Ethyl	$CH_3CH_2NHCOOC_2H_5$	L	23	17. 04			8. 52				. 85
H	*n*-propyl	$CH_3(CH_2)_2NHCOOC_2H_5$	L	23				7. 42				. 74
H	*iso*-propyl	$(CH_3)_2CHNHCOOC_2H_5$	C	23	(*Tripneustes*)			7. 37		. 74		. 07
Diethyl		$(CH_3CH_2)_2NCOOC_2H_5$	L	23		13. 13		6. 56				. 66
H	*n*-butyl	$CH_3(CH_2)_3NHCOOC_2H_5$	L	23			6. 32	. 63				. 06
Pentamethylene			G	26	25. 00			2. 5				. 25
H	Cyclohexyl	$C_6H_{11}NHCOOC_2H_5$	A	26	5. 85		1. 17			. 12		. 01
H	Phenyl	$C_6H_5NHCOOC_2H_5$	D	23	[2] . 61				. 06			. 006
Di-*n*-propyl		$(CH_3CH_2CH_2)_2NCOOC_2H_5$	L	23	5. 43							. 54
Di-*iso*-propyl		$[(CH_3)_2CH]_2NCOOC_2H_5$	L	23	5. 36							. 54
H	*n*-octyl	$CH_3(CH_2)_7NHCOOC_2H_5$	G	26	17. 93	1. 79 . 45		. 18		. 04		. 005
Di-*n*-butyl		$[CH_3(CH_2)_3]_2NCOOC_2H_5$	L	23	. 50							. 05
H	*n*-decyl	$CH_3(CH_2)_9NHCOOC_2H_5$	G	26							14. 78 . 04	
Diphenyl		$(C_6H_5)_2NCOOC_2H_5$	L	23		[2] . 42						. 04
H	2-fluorene	$C_6H_4CH_2C_6H_3NHCOOC_2H_5$	H	23	. 04		. 004				. 0004	

[1] Simultaneous tests.

[2] 1 percent ethanol.

TABLE 3.—*Diurethans:* $C_2H_5OOCNH-R'-NHCOOC_2H_5$

R'	Formula	Source	C°	Millimolarity required to induce—						
				Blocking	Retardation					No effect
				First cleavage	First cleavage			Second cleavage	Embryo	
				Complete	100%	10–99%	<10%			
LYTECHINUS										
None	$(C_2H_5OOCNH)_2$	G	26			45. 44			22. 72	11. 36
Methylene	$(C_2H_5OOCNH)_2CH_2$	L	[1] 23			10. 52			5. 26	
Ethylidene	$(C_2H_5OOCNH)_2CHCH_3$	L	[1] 23			9. 80		4. 90		. 49
Ethylene	$(C_2H_5OOCNH)_2CH_2CH_2$	L	[1] 23			9. 80 4. 90				. 49
Propylene	$(C_2H_5OOCNH)_2(CH_2)_3$	G	[2] 26			18. 35			9. 18	4. 58
Tetramethylene	$(C_2H_5OOCNH)_2(CH_2)_4$	G	[2] 26			8. 62			4. 31	
Hexamethylene	$(C_2H_5OOCNH)_2(CH_2)_6$	G	[2] 26					3. 85		1. 93
TRIPNEUSTES										
None	$(C_2H_5OOCNH)_2$	G	[3] 26					11. 36		5. 68
Propylene	$(C_2H_5OOCNH)_2(CH_2)_3$	G	[3] 26			18. 35 9. 17 4. 58			0. 46	
Tetramethylene	$(C_2H_5OOCNH)_2(CH_2)_4$	G	[3] 26		17. 24	8. 62	4. 31 . 43			. 04
Hexamethylene	$(C_2H_5OOCNH)_2(CH_2)_6$	G	[3] 26			7. 69 3. 85				. 38
o-phenylene	$(C_2H_5OOCNH)_2C_6H_4$	G	[4] 26			[6] 3. 97 [5] . 79				. 08
m-phenylene	$(C_2H_5OOCNH)_2C_6H_4$	G	[4] 26	0. 08	. 008	. 004				. 0004
p-xylylene	$(C_2H_5OOCNHCH_2)_2C_6H_4$	G	[4] 26			[5] . 71	[6] 3. 57			. 07
2,7-fluorene	$(C_2H_5OOCNHC_6H_3)_2CH_2$	H	26			. 29	. 03			[6] . 03

[1], [2], [3], [4] Simultaneous tests. [5] 1 percent ethanol. [6] Direct solution in sea water rather than dispersion by means of ethanol.

TABLE 4.—Bis- *and* Tris*carbamates: 2 or 3 N-substituted carbamic acids esterified on a single phenyl ring: R'R''NCOO–R–OOCNR'R''*

R	R''	R'	Source	Formula	Millimolarity required to induce—						
					Blocking		Retardation				No effect
					First cleavage		First cleavage		Second cleavage	Embryo	
					Complete	Incomplete	10-99%	<10%			
TRIPNEUSTES 23° C											
p-phenyl	H	Methyl	B	$(CH_3NHCOO)_2C_6H_4$	0. 45		0. 04				0. 004
o-phenyl	H	Methyl	D	$(CH_3NHCOO)_2C_6H_4$	[2]. 45	0. 04					. 004
p-phenyl	Dimethyl		D	$[(CH_3)_2NCOO]_2C_6H_4$							1. 99
m-phenyl	Dimethyl [1]		D	$[(CH_3)_2NCOO]_2C_6H_4$			3. 97				[2]. 40
1,2,3-phenyl	Dimethyl [1]		D	$[(CH_3)_2NCOO]_3C_6H_3$				2. 95	0. 30		. 03
LYTECHINUS 23° C											
o-phenyl	H	Methyl	D	$(CH_3NHCOO)_2C_6H_4$			0. 45			0. 04	0. 004
p-phenyl	Dimethyl		D	$[(CH_3)_2NCOO]_2C_6H_4$							[2]. 40

[1] Simultaneous tests.

[2] 1 percent ethanol.

TABLE 5.—*Thiocarbamates*

LYTECHINUS

Name	Formula	Source	C°	Millimolarity required to induce—						
				Blocking		Retardation				No effect
				First cleavage		First cleavage		Second cleavage	Embryo	
				Complete	Incomplete	10-99%	<10%			
	Thiol									
Ethyl thiolcarbamate	$NH_2COSC_2H_5$	L	23	19. 04	------	9. 52	------	------	0. 95	------
	Thiono									
Ethyl thionocarbamate	$NH_2CSOC_2H_5$	L	23	9. 52	------	------	------	------	. 95	------
Ethyl N-ethyl-thionocarbamate	$C_2H_5NHCSOC_2H_5$	G	26	2. 88	------	------	------	------	. 29	------
Ethyl N-phenyl-thionocarbamate	$C_6H_4NHCSOC_2H_5$	G	26	[1] 1. 11	0. 22	{ . 11 . 06 }	------	------	------	0. 01
	Dithio									
Ethyl N,N-diethyl-dithiocarbamate	$(C_2H_5)_2NCSSC_2H_5$	J	26	2. 31	------	------	------	0. 23	------	------
Sodium N,N-diethyl-dithiocarbamate	$(C_2H_5)_2NCSSNa$	------	23	------	------	11. 70	5. 85	------	------	------

[1] 1 percent ethanol.

Amino-substituted Urethan

With the exception of N,N-dimethyl urethan, effectiveness increased with the size of the aliphatic group substituted on the nitrogen (table 2). The aliphatic series up to butyl are easily compared in the column for 10–99 percent delay, and dipropyl to dibutyl in the column for complete block. As with the aliphatic esters, the optimum number of carbons was 10. Octyl urethan produced an incomplete block at 0.45 mM, and the di-*n*-butyl urethan blocked completely at 0.50 mM. At 12 carbons (all counts include the 2 carbons of the ester ethyl) potency dropped sharply in the alkyl series (decyl urethan).

Configuration of the saturated chain was without any considerable influence. Pentamethylene urethan, *i. e.*, a 5-carbon chain linked at both ends to the nitrogen forming a heterocyclic ring, $CH_2(CH_2CH_2)_2NCOOC_2H_5$, fits into its place in the 10–99 percent delay column. Cyclohexyl urethan is more potent (100 percent retardation at 1.17 mM) than pentamethylene, and about comparable to the dipropyls, all blocking at about 5½ mM.

By contrast, the aromatic configuration is important. Phenyl urethan, blocking at 0.61 mM and retarding at 0.06 mM is far more active than cyclic or straight-chain homologues with the same number of carbon atoms (cyclohexyl and dipropyls). N,N-diphenyl urethan maintained a high level of activity, incompletely blocking cleavage at 0.42 mM despite its 14 carbons, while fluoryl urethan, with 15 carbons was the most active carbamate tested.

At this point one can compare several configurations in the isomers *n*-propyl-, *iso*propyl-, ethyl-N-methyl-, and methyl-N,N-dimethyl-carbamate, which all have the same molecular weight. The first retarded cleavage 100 percent and blocked all further development at 19.40 mM. The second only slightly delayed cleavage at this concentration. Ethyl-N-methyl carbamate (*i. e.*, methyl attached to urethan) caused about a 10 percent delay of first cleavage at 9.99 and 19.98 mM. Methyl-N,N-dimethyl merely retarded the later stages of development at 9.7 and 19.4 mM. In this series the unbranched straight chain was the most effective configuration for 3 carbons. These were all tested on *Lytechinus*, methyl-N,N-dimethyl at 26°, and the others at 23°.

Diurethans

The diurethans are comparable to the N-substituted series, except that two molecules are joined directly (diurethan: $C_2H_5OOCNHNHCOOC_2H_5$) or by means of a connecting group (ethylene diurethan: $C_2H_5OOCNHCH_2$-$CH_2NHCOOC_2H_5$). These were tested on *Lytechinus* in two series: methylene, ethylene, and ethylidene at 23° in Bermuda, and propylene, tetramethylene, and hexamethylene at 26° in Bimini. There was consistency within each series, an increasing potency with increasing number of carbons (table 3). Ethylene slowed the first cleavage 13 percent at 4.90 mM and ethylidene was able to retard the second cleavage at 4.90 mM, whereas methylene touched only later stages at 5.26 mM. The weakest was simple diurethan, which has no connecting carbon. It

showed delayed effect at 22.72 mM. In the second series propylene had no effect at 4.58 mM. Tetramethylene retared the gastrulae at 4.31 mM, and hexamethylene slowed the second cleavage at 3.85 mM. The second series gave similar results with *Tripneustes*. Diurethan slowed the second cleavage at 11.36 mM. Propylene diurethan increased first cleavage time by 71 percent at 18.35 mM and 13 percent at 9.17 mM. The increase induced by tetramethylene was 400 percent at 17.24 mM, 32 percent at 8.62 mM and 6 percent at 4.31 mM. Hexamethylene near saturation (3.85 mM) retarded the first cleavage 24 percent. Adding an excess (7.69 mM) merely increased the delay to 32 percent.

When an aromatic ring served as the connecting link there was far more influence exerted by the position of attachment (*o* vs. *m* phenylene, *Tripneustes*, table 3) than by changes in chemical structure (xylene and fluorene).

The impotence of the urethan molecule is underlined by results with diurethans. Doubling the molecule was much less effective than increasing the molecule by a simple 3-carbon chain on the nitrogen or carboxyl oxygen (tables 1 and 2). An additional carbamate moiety considerably lowered the activity of 2-fluoryl urethan.

*Bis*carbamates and *Tris*carbamates

Double and triple molecules joined by the carboxyl group to one phenyl ring are compared in table 4. In this series the N-methyl carbamic acid $(CH_3NHCOO)_2C_6H_4$, proved somewhat stronger when paired in the *ortho* position than when in the *para*. The *ortho* produced an incomplete block at 0.04 mM, whereas the *para* merely caused delay. Data for the dimethyl congeners, $[(CH_3)_2NCOO]_2C_6H_4$, suggest that the *para*, having no effect at 1.99 mM, is weaker than the *meta*. The triple dimethyl congener, $[(CH_3)_2NCOO]_3C_6H_3$, esterified on carbons 1, 2, and 3, was slightly more active than *meta* (delayed effect at 0.30 mM) and considerably more active than *para*.

It is also possible to compare these double compounds with their diurethans of the same molecular weight. The *m*-phenylene diurethan, $C_6H_4(NHCOOC_2H_5)_2$, (table 3) fell into the same range of activity as its inverted *meta* isomer: $[(CH_3)_2NCOO]_2C_6H_4$. One might also include the comparable single molecule, N,N-dimethyl phenyl carbamate, which blocked *Tripneustes* eggs at 12.1 mM, retarded 100 percent at 6.05 mM, and still exerted a delayed effect at 0.61 mM. As with the diurethans, addition of the second carbamate nucleus did not appreciably enhance the antimitotic activity.

Thiocarbamates

Both thiol- and thionourethans were more effective mitotic poisons than urethan (tables 2 and 5). N-ethyl-thionourethan was more active than N-ethyl-urethan, but N-phenyl urethan and the thiono homologue were of about equal potency. N,N-diethyldithiourethan proved to be more potent than N,N-diethyl-urethan.

Derivatives of Phenyl Urethan

In view of the effectiveness of ethyl-N-phenyl carbamate, it was thought worthwhile to examine related compounds. The isomer, phenyl-N,N-dimethyl carbamate was much less active. When tested on *Tripneustes* at 26° C, it retarded cleavage 25 percent at 6.05 mM and required 12.1 mM to block division. Isopropyl-N-phenyl-carbamate was as effective as ethyl-N-phenyl, blocking at 0.56 mM and asserting a delayed effect at 0.06 mM. Octyl-N-phenyl- and octyl-N-cyclohexyl-carbamate, retarding cleavage less than 10 percent at 0.80 and 0.78 mM, respectively, were about a tenth as active as ethyl-N-phenyl-carbamate.

Agreement of Response in the Two Species

Whenever possible, closely related congeners were run simultaneously, or at least they were all tested on a single species. This work has been spread over 2 years and was done at two localities, however, necessitating the use of two species. Compounds tested on both *Lytechinus* and *Tripneustes* or on one species at both localities enable us to evaluate the variables thus introduced. Where the two species were tested at the same temperature and same concentration (table 6), *Lytechinus* was the more disrupted by four compounds, and *Tripneustes* by six. Moreover, the differences are within the range of individual variability for one species, as shown in table 7, so the differences can probably be laid to

Table 6.—*Comparison of effects of similar concentrations on* Tripneustes *and* Lytechinus *at 2 temperatures*

Carbamate	Species	C°	Millimolarity	Percent delay first cleavage
Ethyl-carbamate	L	23	22.4	175
	T	23	56.2	110
	L	26	44.9	90
	T	26	44.9	50
Ethyl-N-ϕ-carbamate	L	23	.06	9
	T	23	.06	22
Methyl-carbamate	L	23	26.66	(1)
	L	26	26.66	(2)
	L	26	106.64	130
	T	26	106.64	128
Diurethan	L	26	11.36	(2)
	T	26	11.36	(3)
Propylene-diurethan	L	26	18.35	24
	T	26	18.35	71
	T	26	9.17	13
Tetramethylene-diurethan	L	26	8.62	90
	T	26	8.62	32
Hexamethylene-diurethan	L	26	3.85	(3)
	T	26	3.85	24
Ethyl-N-methyl-carbamate	L	23	19.98	12
	L	26	37.6	94
	T	26	38.2	34
Ethyl-N-(2-fluoryl)-carbamate	L	23	.0004	(4)
	T	23	.0004	150
o-Phenylene-*bis*-(N-methyl-carbamate)	L	23	.45	32
	T	23	.04	(1)

[1] Incomplete block.
[2] No effect.
[3] Only second cleavage retarded.
[4] Cleavage not delayed.

TABLE 7.—*Response of different lots of eggs to the same concentration of carbamate*

	C°	mM/L	Expt.	Response
Lytechinus				
Ethyl carbamate	26	22. 4	I	No retardation of first cleavage.
	26	22. 4	II	6 percent retardation of first cleavage.
	26	22. 4	III	2 percent retardation of first cleavage.
	26	44. 9	I	90 percent retardation of first cleavage.
	26	44. 9	II	8 percent of the eggs divided 95 min. after fertilization.
	26	44. 9	III	8 percent of the eggs divided 95 min. after fertilization.
Ethyl-N-phenyl-thiono-carbamate.	26	. 11	I	36 percent retardation of first cleavage.
	26	. 11	II	43 percent retardation of first cleavage.
o-Phenylene-*bis*-(N-methyl-carbamate).	23	. 45	I	4 percent retardation of first cleavage.
	23	. 45	II	30 percent retardation of first cleavage.
Tripneustes				
2,7-fluoryl-diurethan	26	. 03	I	No retardation of first cleavage.
	26	. 03	II	4 percent retardation of first cleavage.
	26	. 29	I	10 percent retardation of first cleavage.
	26	. 29	II	16 percent retardation of first cleavage.
Ethyl-N-(2-fluoryl)-carbamate.	23	. 04	I	Incomplete block.
	23	. 04	II	Complete block.

variability rather than any peculiar susceptibility of one species to various members of the carbamate group. This variability also points up the unreliability of small differences in the effects of compounds as reported here, except where it is indicated that they were determined in simultaneous runs on eggs from one urchin.

DISCUSSION

The effort in this series has been directed toward mapping the general trend of effectiveness in a large series of compounds, using only roughly quantitative criteria. More exact dose-effect relationships correlated with cytologic responses of a few compounds will be reported later.

As briefly reported earlier (*33*) this method is sufficient to establish the increase of antimitotic potency with increase in the alkyl ester or N-substituted chain. This fits the schemes correlating liposolubility with mitotic inhibition as elaborated by Östergren and Levan (*12*) and by Gavaudan, Dodé, and Poussel (*13*). Just such a trend is to be expected if the response of mitosis is an indicator of narcosis. Moreover, the actual narcotic effect on mice is deeper with the longer single carbon chain (*2*).

Too inclusive a generalization as to narcotic parallelism must be held in abeyance at present, however. Table 6 shows that urethans were less effective at higher temperatures. Since variables intrinsic in the different localities also were at work, a few series were run at Bimini simultaneously at two temperatures. Here, too, with temperature the only known variable, increase in temperature decreased the urethan retardation of cleavage. Inasmuch as this is the reverse of temperature effect on bacteriophage inactivation (*34*) or on narcosis of bacterial luminescence

(*35*), the relations between temperature and urethan inhibition of cleavage will receive careful further study to determine whether they operate within the narcotic mechanism or merely reflect different rates of urethan decomposition.

Lethality to mice of esters and N-substitutions increases with the length of the chain also, although N-*n*-butyl urethan is less toxic than its molecular weight would seem to justify (*14*). Urethan and ethyl-N-ethyl carbamates are less toxic to mice and to eggs than their thiono homologues. Ethyl-N-β-hydroxyethyl carbamate is the least active of all urethans tested on eggs, and Skipper *et al.*, have shown it to have relatively low toxicity for mice (*14*).

When we compare this antimitotic series with lung tumor production or leukopenic action in mice, however, all parallel disappears. Any ester other than ethyl is less carcinogenic (*2*) and less leukopenic (*14*). Only a few of the N-substituted urethans are comparable to urethan in increasing the numbers of lung tumors. Apart from ethyl-N-*iso*propyl-carbamate, tumor induction decreases with increase in the alkyl chain (*36*). Conversely, methylene and ethylidene diurethans are high in carcinogenicity (*37*) and low in antimitotic activity. Several N-substituted urethans show leukopenic activity, among them, N-phenyl, N,N-dipropyl, and N,N-dibutyl urethan (*14*) which all block sea-urchin mitoses at low concentrations, but the leukopenic and antimitotic seriations appear to be different. Larsen (*38*) and Cowen (*4*) have shown there is no parallel between narcotic and carcinogenic capacity in anesthetics other than urethans.

The carbamate most active against leukemia (*15*) and carcinoma (*9*) found so far is ethyl urethan, one of the weakest from the antimitotic standpoint.

CONCLUSIONS

The potency of carbamates as mitotic poisons shows good correlation with their narcotic and toxic effects, but no correlation with their ability to induce lung tumors or to inhibit leukemia or carcinoma. Accordingly, it seems improbable that the basis of the carcinogenic and carcinoclastic properties of urethan derive from a general nonspecific inhibition of cell division. The possibility of selective damage to malignant mitoses can better be discussed in an ensuing paper on cytologic effects of carbamates.

SUMMARY

1. The cleavage of eggs of the sea urchins *Lytechinus variegatus* and *Tripneustes esculentus* is retarded by carbamates at low concentrations and completely inhibited at high concentrations.

2. In carbamates of the form H_2NCOOR, where R is an alkyl group, potency increases with increase in the number of carbons, up to 10.

3. (a) Alkyl substitutions on the amino group of urethan ($R'R''NCOOC_2H_5$) are also more potent as the number of carbons is increased, up to 8, or 10 including the ester carbons.

(b) Configuration of the alkyl group, whether double, branched, or cyclic, has little effect on its potency.

(c) N-aryl substitutions (R′ or R′R″) are particularly potent.

4. (a) Diurethans ($C_2H_5OOCNHR'NHCOOC_2H_5$) increase in potency as the length of the connecting R′ alkyl group increases.

(b) Aromatic linking groups are more potent than alkyl.

(c) The *meta* position yields a more potent phenylene diurethan than does the *ortho*.

5. (a) Double carbamates of the form R′R″NCOOROOCNR′R″ linked by a benzene ring (R) are consistently more active when there is a total of 8 ester and amino carbons (R′=CH_2, R″=H) than when there are 10 (R′=CH_2, R″=CH_2).

(b) In these compounds the point of attachment on the ring, or even addition of a third urethan nucleus, $C_6H_3[OOCN(CH_3)_2]_3$, exerts relatively little influence.

6. Thiourethans are more active than their oxygen homologues.

7. Within this group of carbamates, the inhibitory effects on mitosis generally parallel their narcotic and toxic potency in mice, but do not correlate with carcinogenic, leukopenic, or antileukemic activity.

8. This wide divergence between carbamates which efficiently inhibit egg cleavage and those which induce tumors or check leukemia leads to the conclusion that generalized suppression of cell division is not the mechanism basic to carcinogenesis or to carcinoclasis by carbamates.

REFERENCES

(*1*) Nettleship, A., and Henshaw, P. S.: Induction of pulmonary tumors in mice with ethyl carbamate (urethane). J. Nat. Cancer Inst. 4: 309–319 (1943).

(*2*) Larsen, C. D.: Evaluation of the carcinogenicity of a series of esters of carbamic acid. J. Nat. Cancer Inst. 8: 99–101 (1947).

(*3*) Orr, J. W.: The induction of pulmonary adenomata in mice by urethane. pp. 311–316. The histology and histogenesis of pulmonary adenomata of mice. pp. 316–322. Brit. J. Cancer 1: 311–322 (1947).

(*4*) Cowen, P. N.: Some studies on the action of urethane on mice. Brit. J. Cancer. 1: 401–405 (1947).

(*5*) Selbie, F. R., and Thackry, A. C.: Lung adenomas induced by urethane in CBA mice. Brit. J. Cancer 2: 380–385 (1948).

(*6*) Smith, W. E., and Rous, P.: The neoplastic potentialities of mouse embryo tissues. IV. Lung adenomas in baby mice as a result of prenatal exposure to urethane. J. Exper. Med. 88: 529–554 (1948).

(*7*) Jaffé, W. G.: Carcinogenic action of ethyl urethane on rats. Cancer Research 7: 107–112 (1947).

(*8*) Guyer, M. F., and Claus, P. E.: Tumor of the lung in rats following injections of urethane (ethyl carbamate). Cancer Research 7: 342–345 (1947).

(*9*) Haddow, A., and Sexton, W. A.: Influence of carbamic esters (urethanes) on experimental animal tumours. Nature 157: 500–503 (1946).

(*10*) Paterson, E., ApThomas, I., Haddow, A., and Watkinson, J. M.: Leukaemia treated with urethane compared with deep X-ray therapy. Lancet 1: 677–682 (1946).

(*11*) Heilmeyer, L., Merk, R., and Pirwitz, J.: Klinik und Pharmakologie des Urethans und anderer cytostatischer Stoffe. Beihefte zur Med. Monatschr. (4) 120 pp. (1948).

(*12*) Östergren, G., and Levan, A.: The connection between c-mitotic activity and water solubility in some monocyclic compounds. Hereditas. 29: 496–498 (1943).

(*13*) Gavaudan, P., Dodé, M., and Poussel, H.: Contribution à l'étude du mecanisme de la narcose par les narcotiques indifférents. Trav. scient. de la Station d'Essais du Bouchet. 2: 1–36 (1946).

(*14*) Skipper, H. E., Bryan, C. E., Riser, W. H., Jr., Welty, M., and Stelzenmuller, A.: Carbamates in the chemotherapy of leukemia. II. The relationship between chemical structure, leukopenic action, and acute toxicity of a group of urethan derivatives. J. Nat. Cancer Inst. 9: 77–88 (1948).

(*15*) Skipper, H. E., and Bryan, C. E.: Carbamates in the chemotherapy of leukemia. III. The relationship between chemical structure and anti-leukemic action of a series of urethan derivatives. J. Nat. Cancer Inst. 9: 391–397 (1949).

(*16*) Boyland, E.: Chemical carcinogenesis and experimental chemotherapy of cancer. Yale J. Biol. & Med. 20: 321–341 (1948).

(*17*) Haddow, A., and Robinson, A. M.: The influence of various polycyclic hydrocarbons on the growth rate of transplantable tumours. Proc. Roy. Soc. London, s. B 122: 442–476 (1937).

(*18*) ———: The association of carcinogenicity and growth-inhibiting power in polycyclic hydrocarbons and other substances. Proc. Roy. Soc. London, s. B 127: 277–287 (1939).

(*19*) Haddow, A.: The influence of carcinogenic compounds and related substances on the rate of growth of spontaneous tumors of the mouse. J. Path. & Bact. 47: 567–579 (1938).

(*20*) Haddow, A., Scott, C. M., and Scott, J. D.: Influence of certain carcinogenic and other hydrocarbons on body growth in rat. Proc. Roy. Soc. London, s. B 122: 477–507 (1937).

(*21*) Haddow, A., Harris, R. J. C., and Kon, G. A. R.: Inhibition of growth by amino-*s*-diarylethylenes. Biochem. J. 39: Proceedings ii (1945).

(*22*) Badger, G. M., Elson, L. A., Haddow, A., Hewett, C. L. and Robinson, A. M.: The inhibition of growth by chemical compounds. Proc. Roy. Soc. London, s. B 130: 255–299 (1942).

(*23*) Elson, L. A.: The effect of diet on the body-growth- and tumor-growth inhibiting action of carcinogenic substances. 4th Internat. Cancer Research Cong. (Abstract) p. 66 (1947).

(*24*) Haddow, A., Harris, R. J. C., Kon, G. A. R., and Roe, E. M. F.: The growth-inhibitory and carcinogenic properties of 4-aminostilbene and derivatives. 4th Internat. Cancer Research Cong. (Abstract) p. 20 (1947).

(*25*) Lettré, H.: Über das Wachstum des Mäuse- Ascites- Tumors und seine Beeinflussung. Angew. Chemie. 54: 202 (1941).

(*26*) Seligman, A. M., Friedman, O. M., Gofstein, R. M., and Sweet, B. H.: Mouse tumor growth inhibition with compounds related to the carcinogenic hydrocarbons. 4th Internat. Cancer Research Cong. (Abstract) p. 90 (1947).

(*27*) Stamer, S.: Effect of carcinogenic hydrocarbon on manifest malignant tumors in mice. Acta path. et microbiol. Scandinav., Supp. 47: 11–158 (1943).

(*28*) Stern, K., and Willheim, R.: The biochemistry of malignant tumors. Reference Press (1943).

(*29*) Ferrari, S.: Azioni del 3:4 benzopirene sull 'attecchiemento e l'evoluzione dell adenocarcinoma di Ehrlich. Patologica. 40: 221–223 (1948).

(*30*) Sdruikovskaya, A. I.: On the effect of cancerogenic substances on the healing of skin wounds in mammals. C. R. Acad. Sci. URSS. 30: 870–873 (1941).

(*31*) Warburg, O.: Über die Oxydationen in lebenden zellen nach Versuchen am Seeigelei. H-S Zschr. Physiol. Chem. 66: 305–340 (1910).

(*32*) Cornman, I: The action of podophyllin and its fractions on marine eggs. Annals N. Y. Acad. Sci. (In press.)

(*33*) ———: Inhibition of sea urchin egg cleavage by a series of substituted carbamates. Biol. Bull. 95: 244 (1948).

(*34*) JOHNSON, F. H., and FOSTER, R. A. C.: Influence of hydrostatic pressure and of urethan on the thermal inactivation rate of bacteriophage. Fed. Proc. 8: 82 (1949).

(*35*) JOHNSON, F. H., EYRING, H., and WILLIAMS, R. W.: The nature of enzyme inhibitions in bacterial luminescence: sulfanilamide, urethane, temperature and pressure. J. Cell. & Comp. Physiol. 20: 247–268 (1942).

(*36*) LARSEN, C. D.: Studies of pulmonary tumor induction in mice by derivatives of carbamic acid. Cancer Research 7: 726 (1947).

(*37*) ———: Pulmonary tumor induction with alkylated urethans. J. Nat. Cancer Inst. 9: 35–37 (1948).

(*38*) ———: Survey of hypnotics in general with regard to pulmonary-tumor induction in mice. J. Nat. Cancer Inst. 7: 5–8 (1946).

FACTORS IN THE DEVELOPMENT OF SPONTANEOUS MAMMARY GLAND TUMORS IN AGENT-FREE STRAIN C_3Hb MICE [1]

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Mice do not become infected with the mammary tumor-agent *in utero*, but infection occurs from the milk of infected mothers. It is to be expected, therefore, that mice taken from the mother by cesarean section and nursed upon a foster mother of a low-tumor strain will not have the mammary tumor-agent even though the natural mother was of a high-mammary-tumor strain and had the agent. Such procedure is commonly accepted for starting lines to be used in testing for the agent.

From a litter taken by cesarean section and nursed upon a low-tumor strain foster mother, a line of strain C_3H mice free of the agent was developed in this laboratory. This line is designated as C_3Hb. It was anticipated that in the absence of the mammary tumor agent there would be a low incidence of mammary gland tumors in the line. On the contrary, however, a relatively high incidence has occurred. An analysis of factors in the development of these tumors and morphologic descriptions of them are presented in this paper. A preliminary brief report on the C_3Hb line has been published (*1*).

ORIGIN OF LINE C_3Hb

Line C_3Hb was derived from the line of strain C_3H mice maintained in the genetics laboratories of the National Cancer Institute. This C_3H line was separated from the colony of Dr. H. B. Andervont in 1941, and he had obtained his line from Dr. L. C. Strong in 1930. The incidence of mammary gland tumors in the breeding females of our strain C_3H colony, which has included 419 females, has been recorded recently as 96.7 percent with an average tumor age of 7.8 months. The average age at which the 3.3 percent died without mammary tumors was 6.9 months. Forty-six females of this line were kept as virgins, and all of these developed mammary gland tumors at an average age of 10.6 months.

In 1945, line C_3Hb was started from a litter of three females and three males that was removed from a strain C_3H mother by cesarean section

[1] Received for publication January 12, 1950.

and foster nursed upon a low-tumor strain C57 black female. This foster mother was from the C57 black colony maintained in our laboratories. In this colony no mammary gland tumors have been observed in a total of 198 breeding females. The average age at which these females died or were killed and examined was 13.8 months. Other investigators (*2*) have reported an incidence of less than 1 percent for the strain. Experimental work with this strain has never disclosed the presence of the mammary tumor-agent.

The three females of the original C_3Hb litter were mated to one of their male littermates and in subsequent generations the line has been maintained by brother × sister matings, usually littermates. With the exception of the original litter the young in the line have been nursed by their natural mothers.

TUMOR INCIDENCE

One hundred eighty-eight breeding females from the first five generations of the line are included in this report. All these females were permitted to have as many litters as possible. They were kept in wooden cages, allowed constant access to tap water, and given a continuous supply of a diet, the formula of which has been published by Morris (*3*).

An autopsy was done on each animal after a tumor appeared or when the animal became moribund or was found dead. The tumors were fixed in Tellyesniczky's fixative (70 percent ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part), and were stained with hematoxylin and eosin.

Of the original three females, No. 23388, which with her descendants constitutes line *I*, developed a mammary-gland tumor at 18 months of age. She and her female descendants totaled 119 of which 48 or 40 percent developed mammary tumors at an average age of 20.1 months. The remaining 71 died without mammary tumors at an average age of 17.6 months.

The second original female, 23389, which with her descendants constitutes line *II*, did not develop a mammary-gland tumor although she lived to 26 months of age. Mammary-gland tumors did appear, however, in 15 of her 48 descendants. This was an incidence of 31 percent for the line. The average tumor age was 19.8 months and the average age at which the nontumorous females died was 19.8 months. Despite the fact that the original female of line *II* did not develop a mammary tumor whereas that of line *I* did, there was not a statistically significant difference between the incidences of the two lines. $X^2=1.4$; P is between 20. and 30 percent.

The third original female, 23390, the progenitor of line *III*, developed a mammary-gland tumor at 20 months of age. She and her female descendants totaled 20 in this line, of which 8 or 40 percent developed mammary-gland tumors at an average age of 21.3 months. The remaining 12 died at an average age of 18.3 months. This incidence was not significantly different from that of either of the other two lines.

Out of the 188 females of the three lines combined, 71 or 38 percent developed mammary gland tumors at an average age of 20.3 months with a range of from 9 to 26 months. Those without mammary tumors died at an average age of 18.3 months. This incidence can be contrasted with the tumor incidence of 97 percent for the breeding females of the strain C_3H with an average tumor age of 7.8 months ranging from 4 to 14 months and an average nontumor age of 6.9 months, table 1.

Aside from the mammary gland tumors the following neoplasms were found in the C_3Hb females: 9 ovarian tumors, 5 primary lung tumors, 1 hemangioendothelioma in the wall of the uterus, 1 subcutaneous sarcoma, 1 pigmented squamous-cell carcinoma of the skin, 1 sarcoma at the hilum of the ovary, and 9 females had one or more hepatomas each.

TABLE 1.—*Mammary tumor incidence of females of strains C_3H, C57 black and C_3Hb*

Strain	Number	Tumorous		Nontumorous	
		Percent	Average age (month)	Percent	Average age (month)
C_3H (breeders)	419	97	7. 8	3	6. 9
C_3H (virgins)	46	100	10. 6	0	------
C57 black (breeders)	198	0	------	100	13. 8
C_3Hb (breeders)	188	38	20. 3	62	18. 3
C_3Hb (virgins—fostered upon C57 black)	160	4	24. 2	96	23. 0

HISTOPATHOLOGY OF THE MAMMARY-GLAND TUMORS

A rigid classification of mammary-gland tumors in the mouse should not be attempted because of the histologic variation seen in different areas of the same tumor, and because of the gradual merging of one tumor type into another (*4*). On the other hand, when a number of tumors from a uniform animal source are available, and when these can be compared with other large groups of mammary tumors in the mouse, a significant difference in the occurrence of certain histologic features becomes readily apparent. This is especially true when inbred strains with and without the mammary tumor-agent can be compared. In this study a group of 123 mammary tumors from C_3H mice with the mammary tumor-agent were compared with tumors from the agent-free mice. Thirty-eight of the mice with the agent were C_3H, and 85 were C_3Hb reinfected with the agent.

The tumors were separated into the following groups:

1. *Adenocarcinomas.*—These are the most common form of mammary tumors in mice. A reproduction of small mammary acini is usually apparent. The entire tumor section may show this acinar structure, or it may appear in only small areas (fig. 1). Considerable variation may also appear in the development of gland lumens. They may be indistinct, so that a solid structure is reproduced in many areas (fig. 2) or they may be uniformly large and resemble thyroid follicles. The amount of stroma, the number of cystic spaces, and the size of the cells likewise vary; but

the basic acinar structure can be recognized as a rule, and these tumors are readily identified as the commonly encountered neoplasm of mammary origin. One hundred and twenty-one, or more than 98 percent of the tumors from the C_3H mice with the mammary tumor-agent were placed in this group; whereas only 60 or 73 percent of the 82 tumors in mice without the agent were so sorted.

2. *Adenoacanthoma.*—Tumors having a notable degree of squamous-cell elements were placed in this category. Often, more than half of the area of a section showed keratinization (fig. 3). Of particular interest among the tumors in the C_3Hb mice were those of a predominantly acinar structure in which large cysts lined by stratified squamous epithelium were found (fig. 4). Only 1 adenoacanthoma was observed in the mice with the agent, an incidence of less than 1 percent, while 17, or over 20 percent, of the tumors in mice without the agent were of this type.

3. *Miscellaneous.*—These are tumors which could not readily be placed in either of the preceding groups and which differed among themselves, so that they do not form a distinct category. One tumor of a mouse with the agent was composed of solid nests of unusually small cells; it was placed in this group along with five tumors in mice without the agent. These five tumors were described as: (a) two composed of unusually large cuboidal cells lining clefts or cysts, or growing in solid sheets, with little suggestion of an acinar structure (fig. 5); (b) one of solid sheets of epithelial cells without acinar structure; (c) one of multiple large cysts lined by a single layer of cells with some areas of acinar structure in the connective tissue between the cysts; and (d) one in which epithelial cells appeared to merge with sarcomatous elements in the stroma, forming a carcinosarcoma (fig. 6). This last type was described and discussed in a recent paper (*4*).

The numbers of tumors falling within the three groups are given in table 2. The total number of tumors is slightly greater than the total number of mice with tumors given in table 1, because a few animals had more than one tumor.

TABLE 2.—*Classification of mammary tumors of the C_3Hb females*

Type	Number
Adenocarcinoma (typical)	60
Adenoacanthoma (keratinized)	17
Miscellaneous	5
Total	82

It is evident that there is greater histologic variation in the mammary tumors from the C_3Hb mice without the mammary tumor-agent than from strain C_3H mice with the agent. Especially significant was the increase in adenoacanthomas. This type of tumor has been reported by Kirschbaum *et al.* (*5*), as occurring in certain inbred strains and hybrid types of mice following treatment with methylcholanthrene of both

females with the mammary tumor-agent and those without the agent. The histogenesis of these tumors was described as differing from that of the usual mammary-gland tumor developing in the presence of the mammary tumor-agent. More recently Kirschbaum (*6*) has described the same type of tumor occurring in old females of certain hybrid types, presumably without the mammary tumor-agent.

INFLUENCE OF BREEDING

Each of the C_3Hb females was placed with a male sib at weaning age (4 weeks), and when she became pregnant was removed from the breeding cage to an isolation cage. When the litter was weaned the mother was returned to the breeding cage, and this procedure was repeated as long as the female remained fertile. All females bred, the number of litters ranging from one to eight with a mean number of 4.52.

The females that developed mammary tumors had on the average more litters than did the nontumorous females, the mean for the tumor-bearing females being 4.83 while that for the nontumorous females was 4.33. While the difference between these two means is not great, it is real: $t=2.56$; P is between 1 and 2 percent. Certain factors responsible for this difference, however, also may have been influential in causing the age difference. The females that developed mammary tumors lived on the average 2 months longer than did the nontumorous females. However, most of the females had quit breeding several months before death.

A group of C_3Hb females has been set aside as virgins in order to ascertain the incidence of tumors for females of the strain kept as virgins, but the final data for these females are not yet available. It appears, however, that this incidence will be low. To date, not one of these virgin females has developed a tumor although they are approximately a year old or older.

A group of 160 virgin females of the line was used in another experiment in which they were nursed upon strain C57 black females (table 1). Only 6 or 3.75 percent of these females developed a mammary tumor, the mean tumor age being 24.2 months. The remaining 154 died at an average age of 23 months, without having developed a mammary tumor. These virgin females differed from the 185 C_3Hb breeding females of this report in that they nursed upon C57 black females. It should be pointed out, however, that the original three C_3Hb females considered herein also nursed upon a C57 black female and of these three, two developed a tumor.

Of 112 C_3Hb virgin females that were used for testing for the mammary tumor agent as reported in a subsequent section (table 3), only 1 has developed a tumor. Forty-nine of these females are still living without tumors at an average age of 17.6 months. Since the agent apparently was absent, as will be discussed more fully, the data can be considered as giving an indication of the tumor incidence of the virgin females of the line. Thus, the data from the various groups seem to be in agreement, indicating an incidence of less than 5 percent for the C_3Hb females when kept as virgins.

PARENT-OFFSPRING CORRELATION

In search of evidence of the presence of the mammary tumor-agent in the line, and particularly in the females that developed tumors, a study of the distribution of the tumorous females in the pedigree charts was made (text-figures 1, 2, and 3). As can be noted, this distribution fails to give convincing evidence for the presence of the agent. If the agent were to occur in any of the females one would expect her descendants to constitute a high tumor line as compared to the other families in the chart. In general, however, the tumor-bearing females were scattered throughout the charts with no clear-cut segregation of high- and low-tumor lines.

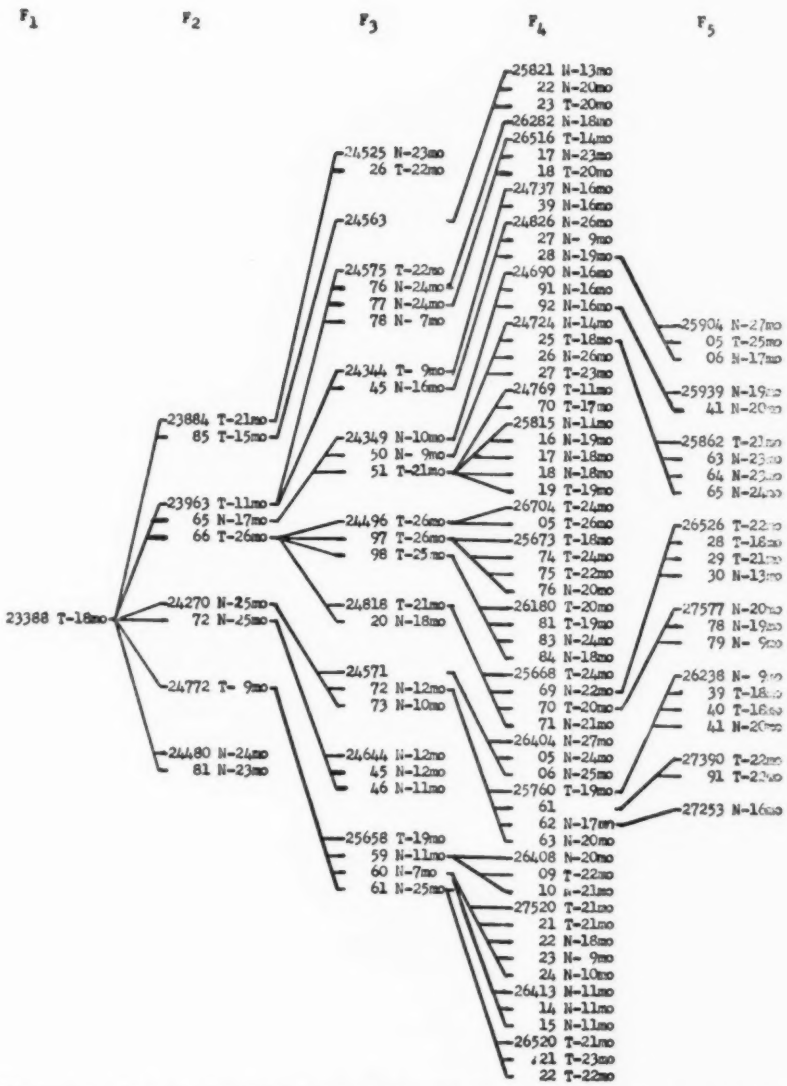
More detailed analysis, however, revealed a slight positive parent-offspring correlation. Among the offspring of tumor mothers, 38 developed tumors and 42 died nontumorous, whereas among the offspring of nontumor mothers, 24 developed tumors and 65 died nontumorous. Thus, the chance that a female would develop a tumor was slightly greater if her mother developed a tumor than if her mother were nontumorous. Although the difference was not great it was statistically significant. $X^2=8.55$; $P<0.01$. This, however, does not constitute proof of the presence of the mammary tumor-agent, because slight variation in genetic factors or in other extrachromosomal factors could be expected to give such a correlation.

TESTS FOR THE PRESENCE OF THE MAMMARY TUMOR-AGENT IN THE C_3Hb TUMORS

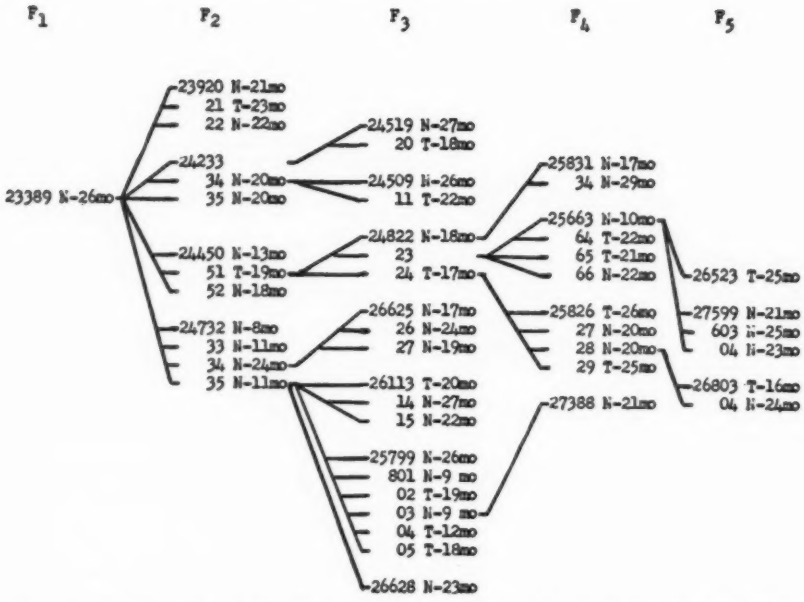
Twenty-four of the mammary-gland tumors that arose in the C_3Hb females were tested for presence of the mammary tumor-agent. Two to five percent extracts of the tumors were prepared in 0.001 M phosphate buffer, pH 7.0, with a Waring blendor; cleared in a multispeed centrifuge at 18,000 x G for 3 minutes; and filtered through a Berkefeld H or an 8 lb. Mandler filter.[3] To test for the agent 0.1 cc. of the cell-free filtrate was injected into C_3Hb females from 1 to 5 days of age. After these females were weaned they were set aside as virgins and were observed for the development of mammary gland tumors. Mammary gland tumors that arose in three C_3H females were tested in the same manner.

Results of these tests are presented in table 3. Of the total of 112 C_3Hb females injected with filtrates from tumors of C_3Hb females, only one developed a mammary-gland tumor. Sixty-two have died nontumorous at an average age of 19.5 months and 49 are still living but without tumors at an average age of 17.6 months, the youngest being 13 months of age. In contrast, of the 12 C_3Hb females injected with the filtrates from the tumors that arose in C_3H females, 9 have developed mammary-gland tumors at an average age of 10.4 months and 3 are still living without tumors at an average age of 13.7 months. Although tests of only 3 C_3H females are recorded here, in our laboratory many mammary tumors

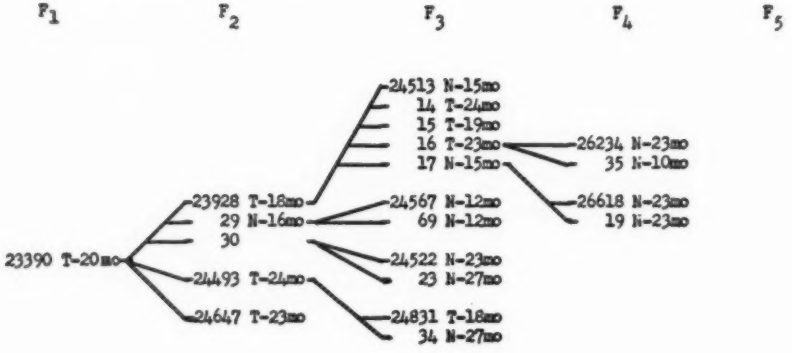
[3] The first 11 of these cell-free filtrates were prepared in Dr. W. R. Bryan's virus laboratory.



TEXT-FIGURE 1.—Pedigree chart of Line I of the C3Hb colony. T = tumor at the age indicated. N = died without tumor at age indicated. Absence of T or N = no final record.



TEXT-FIGURE 2.—Pedigree chart of Line II of the C_3Hb colony. T=tumor at the age indicated. N=died without tumor at age indicated. Absence of T or N=no final record.



TEXT-FIGURE 3.—Pedigree chart of Line III of the C_3Hb colony. T=tumor at the age indicated. N=died without tumor at age indicated. Absence of T or N=no final record.

from C_3H females have been tested in this manner and all have been shown to contain the agent.

TABLE 3.—*Tests for the presence of the mammary tumor agent in mammary tumors from C_3Hb and C_3H females.*

Tumor		Test C_3Hb females						
			Tumorous		Nontumorous			
					Dead		Living	
Female No.	Age tumor arose (month)	Total number test females	Number	Average tumor age (month)	Number	Average age died (month)	Number	Age (month)
C_3Hb females:								
23388 [1]	18	6			4	19.8	2	26
23885 [1]	15	5			3	15.0	2	18
23921	23	4			4	18.3		
23928	18	4			4	19.5		
23963	11	8			8	23.1		
24344	9	7			7	22.2		
24520	18	3					3	24
24575	22	6			1	14.0	5	19
24647	23	6			1	17.0	5	19
24725	18	1					1	24
24769	11	5			5	23.2		
24770	17	5			5	22.0		
24772	9	7			7	22.0		
24818	21	8			8	14.8		
24831	18	2			2	18.0		
25658	19	5	1	12			4	19
25760	19	5					5	19
25862	21	5					5	14
26180	20	2			1	14.0	1	15
26181	19	4					4	15
26518	21	4					4	15
26528	18	5			1	8.0	4	14
26529	21	4			1	8.0	3	13
26803	16	1					1	17
C_3H females:								
23980	8	4	4	9.8				
30556	7	5	3	12.0			2	14
30658	8	3	2	9.0			1	13

[1] Transplant of the original tumor in C_3Hb female tested.

From these data it is evident that of the 24 tumors from C_3Hb females that were tested, none contained the agent such as occurs in mammary tumors of C_3H females. In one set of test females a tumor arose in one, but the remaining four are still living without tumors at 19 months of age, which is well beyond the age at which mammary tumors usually arise in females of this strain with the agent. Furthermore, in consideration of the tumor incidence in noninjected C_3Hb females the occurrence of this one tumor cannot be considered as a positive test for the agent.

DISCUSSION

In the development of mammary-gland tumors in the mouse, many factors, both genetic and nongenetic, can be influential. The tumor

appears when the combined effects of these factors surpass a physiologic threshold. As data accumulate it becomes more evident that no factor or particular potency of a given factor, beyond that necessary for the development of a mouse with a mammary gland, is essential for the appearance of the tumor. The presence or absence or the alteration in potency of a given factor merely alters the probability that the tumor will occur by shifting the level about which the total effect of all factors will fall. Only by controlling the other factors to maintain a constant mean for their total effects can this shift be measured.

Basic among these influencing factors are the genetic factors. In many of the genetic experiments, however, the possibility of variation in other factors has not been ruled out, and thus a measure of the effect of variation in genotype alone has not been shown. Because of variation in these other factors, it is difficult to ascertain just how much of the differences between the incidences of the various inbred strains is owing to direct effect of genetic differences. The direct effect of the genetic difference between strain C_3H and strain C57 black can be measured only when strain C57 black is given the mammary tumor-agent or when the C_3H females are deprived of the agent. In the first situation the effect is represented by the difference between the 97 percent incidence for the C_3H females and the approximately 50 percent incidence for the C57 black females given the agent. In the latter situation the effect is measured by the difference between the 38 percent incidence of the C_3Hb females without the agent and the less than 1 percent incidence of the C57 black females without the agent.

One of the most accurate measures of the effect of genetic variation alone that has been reported was in a project of Heston *et al.* (*7*) Here the genetic difference affected in one generation of backcrossing F_1 females to the susceptible parent strain C_3H male on the one hand and to the resistant parent strain C57 black male on the other resulted in an incidence difference between 93 percent mammary tumors in the susceptible strain backcross females and 55 percent in the resistant strain backcross females. In these backcrosses there was genetic variation within each group, but for each group the incidence represents the mean probability that the individuals would develop a tumor with this particular set of controlled factors as a background.

In certain strains the shift in the probability that a mammary tumor will appear, which is brought about by the additional hormonal stimulation resulting from breeding, is readily seen. In the well known strain A, the incidence of spontaneous mammary-gland tumors in virgin females is less than 5 percent, but when the factor of breeding is introduced this incidence is raised to approximately 70 or 80 percent. Jones (*8*) showed that this incidence could be varied further by additional variation in the breeding factor as represented by variation in number of litters.

In the strain C_3H females in this laboratory the effect on the tumor incidence of breeding the females is not readily discernible. In both the breeders and virgins the incidence is from 95 to 100 percent. The only

difference one can detect in the probability levels of the two groups is the slight difference in tumor age. The average age at which the breeders get tumors is approximately 8 months, whereas that for the virgins is 10.5 months.

As could be expected, however, through the elimination of a very potent factor, in this case the mammary tumor-agent, the level of the total effect of the other factors was brought down to a range in the C_3Hb at which the effect of variation in hormonal stimulation was readily seen. Evidence thus far indicates that the incidence in the virgin C_3Hb females is less than 5 percent. Upon the introduction of the breeding factor—in this case a strong breeding factor, for the females had many litters—this incidence was shifted from less than 5 to 38 percent.

Development of this strain C_3Hb has presented a measure of the shift in probability level brought about by variation in the mammary tumor-agent including its presence and absence. Although it is realized that the evidence presented does not constitute absolute proof that the agent is not present in these C_3Hb mice, it is emphasized that the testing procedures that have always demonstrated the agent in strain C_3H mice failed to give evidence of it in the C_3Hb females. If the mammary tumor-agent is not present, then the 38 percent represents the incidence of tumors one can expect in the conditions presented by this strain in the absence of the agent. Furthermore, the difference between 97 and 38 percent represents the shift in probability level produced by the elimination of the agent.

Results of the application of carcinogenic hydrocarbons also indicate that the mammary tumor-agent is not essential in the development of mammary-gland tumors in the mouse. Here again when the very potent agent is removed the effect of another factor, the hydrocarbon, becomes even more striking. Kirschbaum and coworkers (*5–6*) increased the incidence of, and accelerated the appearance of, mammary tumors in various strains and hybrid types in the absence of the agent as well as in its presence. This has also been effected by Andervont and Dunn (*4*) using strain dba. The results of these and other investigators (*9–11*) in inducing mammary tumors in mice with carcinogenic hydrocarbons make it evident that these substances fall in line with other factors which can influence the probability that the malignant transformation will occur.

Radiation is another factor which when introduced in certain conditions has been noted to raise the probability level that mammary tumors will occur. As reported by Lorenz *et al.* (*12*), when virgin females of this C_3Hb strain were exposed to 8.8 r of gamma radiation for 8 hours daily, 23 percent developed mammary carcinomas at an average tumor age of 15 months. When this incidence is compared with the less than 5 percent incidence of the virgin C_3Hb females, the shift in probability level effected by the radiation appears remarkable. The fact that the incidence in the irradiated virgin females was not as high as that of females bred intensively, although the tumor age was lower, indicates that the shift produced by radiation was no greater than that produced by the intensive breeding.

Finally, one of the factors to be considered is time itself. When the combined effect of all factors is at a low level the average tumor age is greater than when the combined effect is high. Thus, the effect of the removal of the mammary tumor-agent in the development of the C_3Hb strain was not as great as the difference in incidence alone would indicate, for the tumors in the C_3Hb females arose at a much later age.

It is thus apparent that many factors can be influential in raising the cumulative effect in a given female to the threshold at which the malignant transformation occurs, although no single factors or no exact potency of a single factor may be essential. Outstanding in regard to the C_3Hb females is the fact that the incidence of spontaneous mammary-gland tumors is relatively high. The explanation offered is that other factors such as the genotype and the intensive breeding were strong enough to raise the probability to such a level that, given a long period of time, a relatively high incidence of tumors occurred in the absence of the mammary tumor-agent.

A basic difference between the situation represented by this tumor with its related agent or virus and that of communicable diseases should be emphasized. It is true that factors other than the infective organism, such as genetic susceptibility, age, or others, may affect the incidence of typhoid fever or smallpox, but one does not get typhoid fever or smallpox without the bacillus or the virus. In the development of mouse mammary tumors, the mammary tumor-agent may be a very decisive factor, but there is now strong evidence that some mouse mammary tumors occur in the absence of this agent.

SUMMARY

From a litter of high-tumor strain C_3H mice born by cesarean section and foster nursed upon a low-tumor strain C57 black female, a strain designated as C_3Hb and deprived of the mammary tumor-agent has been developed. Of 188 C_3Hb females from the first five generations that have been bred intensively, 38 percent have developed mammary-gland tumors at an average age of 20.3 months.

Greater histologic variation occurred in these mammary tumors from the C_3Hb females without the agent than is encountered in tumors from C_3H females with the agent. The increase in keratinized tumors was especially significant.

By the removal of the mammary tumor-agent the effect that breeding has upon the chance that a female of strain C_3H will develop a mammary tumor became much more discernible. The incidence of mammary tumors in virgin C_3Hb females was less than 5 percent as compared with 38 percent in the breeding females. An incidence of from 95 to 100 percent occurs in both the breeding and virgin C_3H females with the agent.

Progeny of tumor-bearing females did not segregate as high-tumor lines in the colony as might be expected if the mammary tumor-agent were involved. Tests for the presence of the agent in cell-free filtrates of 24 of the tumors from the C_3Hb females have all been negative, although

all comparable tests of mammary tumors from C_3H females have been positive.

It was concluded that other factors such as the genotype of the line and the intensive breeding were strong enough to result in a relatively high incidence of mammary tumors in the C_3Hb females in the absence of the mammary tumor-agent.

REFERENCES

(*1*) HESTON, W. E.: Role of genes and their relationship to extrachromosomal factors in the development of mammary gland tumors in mice. Brit. Cancer 2: 87–90 (1948).

(*2*) LITTLE, C. C., MURRAY, W. S., and CLOUDMAN, A. M.: The genetics of non-epithelial tumor formation in mice. Am. J. Cancer 36: 431–450 (1939).

(*3*) MORRIS, H. P.: Diet and some other environmental influences in the genesis and growth of mammary tumors in mice. Publ. Amer. Assoc. Advancement Sci. 22: 140–161 (1945).

(*4*) ANDERVONT, H. B., and DUNN, T. B.: Response of mammary-tumor-agent-free strain dba female mice to percutaneous application of methylcholanthrene. Nat. Cancer Inst. 10: 895–926 (1950).

(*5*) KIRSCHBAUM, A., WILLIAMS, L., and BITTNER, J. J.: Induction of mammary cancer with methylcholanthrene; histogenesis of the induced neoplasm. Cancer Research 6: 354–362 (1946).

(*6*) KIRSCHBAUM, A.: Induction of mammary cancer with methylcholanthrene; histological similarity between carcinogen induced tumors and certain mammary neoplasms occurring spontaneously. Cancer Research 9: 93–95 (1949).

(*7*) HESTON, W. E., DERINGER, M. K., and ANDERVONT, H. B.: Gene-milk agent relationship in mammary-tumor development. J. Nat. Cancer Inst. 5: 289–307 (1945).

(*8*) JONES, E. E.: Tumor incidence in line A albino mice following injections of progynon B. Am. J. Cancer 39: 94–99 (1940).

(*9*) ENGELBRETH-HOLM, J., and LEFÉVRE, H.: Acceleration of the development of leukemias and mammary carcinomas in mice by 9,10-dimethyl-1,2-benzanthracene. Cancer Research 1: 102–108 (1941).

(*10*) ENGELBRETH-HOLM, J.: Acceleration of the development of mammary carcinomas in mice by methylcholanthrene. Cancer Research 1: 109–114 (1941).

(*11*) LEFÉVRE, H.: Acceleration of the development of spontaneous tumors in mice. Thaning and Appels Forlag. Copenhagen (1945).

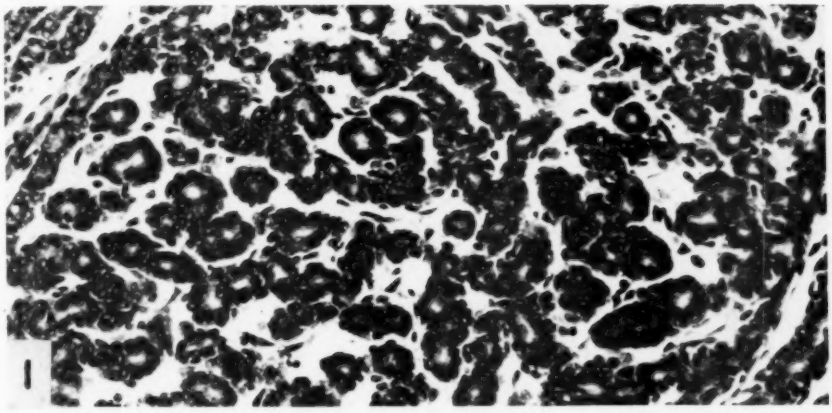
(*12*) LORENZ, E., HESTON, W. E., and ESCHENBRENNER, A. B.: Effects of chronic irradiation with gamma rays on mammary tumor incidence in C_3Hb mice. Cancer Research 9: 621 (1949).

PLATE 177

FIGURE 1.—Adenocarcinoma, reproducing mammary acini. C_3Hb—age 24 months. Hematoxylin and eosin. × 250.

FIGURE 2.—Adenocarcinoma, showing solid nests of cells with little evidence of acinar formation. C_3Hb—age 26 months. Hematoxylin and eosin. × 250.

FIGURE 3.—Adenoacanthoma, with extensive areas of stratified squamous epithelium. C_3Hb—age 23 months. Hematoxylin and eosin. × 65.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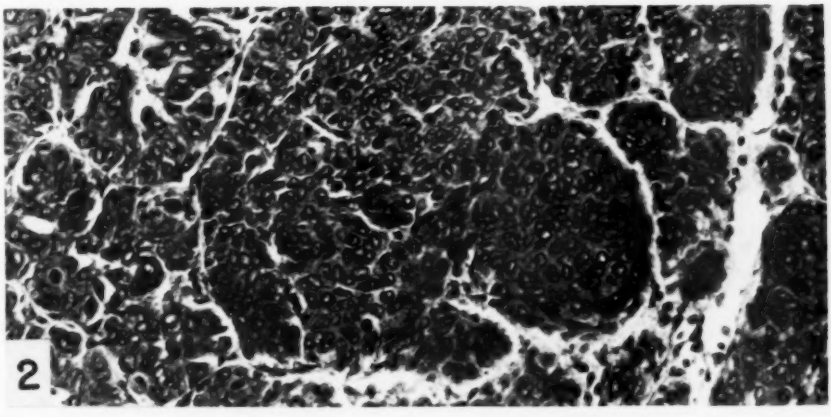
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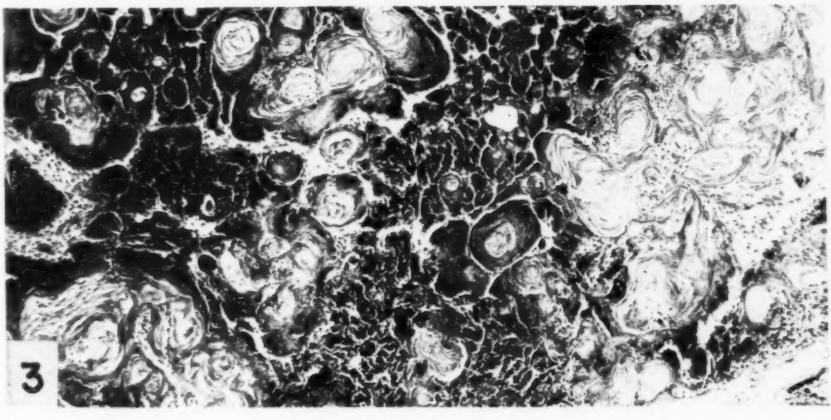
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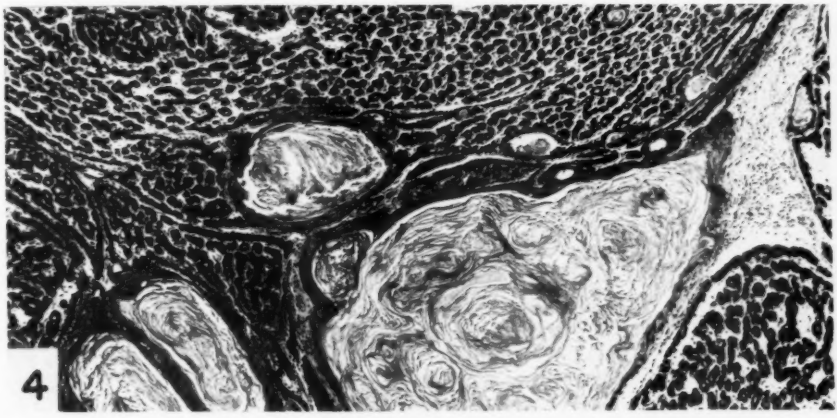
PLATE 178

FIGURE 4.—Adenoacanthoma, showing cysts lined by stratified squamous epithelium, in a tumor of predominantly acinar structure. C_3Hb—age 26 months. Hematoxylin and eosin. × 65.

FIGURE 5.—A "miscellaneous" mammary tumor, showing cuboidal epithelial cells, and no reproduction of small mammary acini. C_3Hb—age 11 months. Hematoxylin and eosin. × 250.

FIGURE 6.—Carcinosarcoma, showing merging of malignant epithelial and stromal tissue. C_3Hb—age 23 months. Hematoxylin and eosin. × 250.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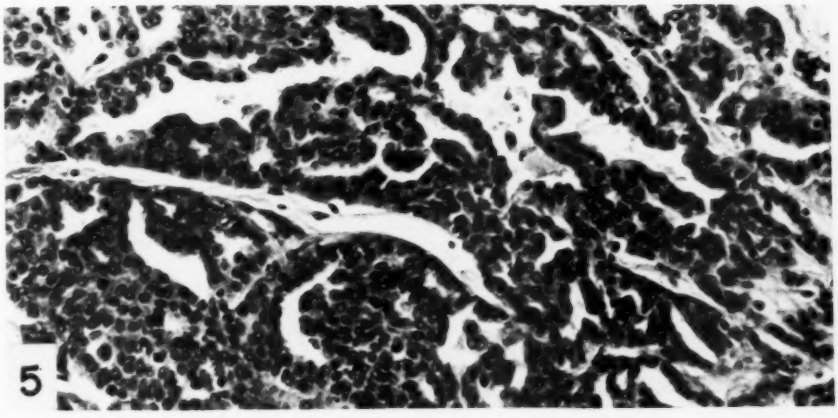
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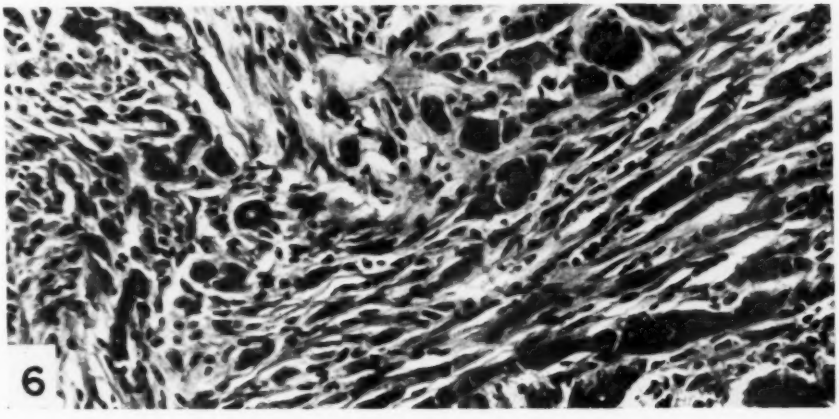
6

ATTEMPT TO DETECT A MAMMARY TUMOR-AGENT IN STRAIN C MICE BY X-RADIATION

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A high incidence of mammary tumors in successive generations of inbred mice is known to be associated with the presence of an agent in mothers' milk. Since the occurrence of these tumors is, presumably, dependent upon the agent, hybrid females derived from low-mammary-tumor-strain females and high-mammary-tumor-strain males show a low incidence of tumors because their mothers do not possess the agent.

An exception to the usual results attending the crossing of two such strains was encountered when F_1 hybrids procured from low-tumor-strain C females and high-tumor-strain C_3H males exhibited a high incidence of mammary tumors (*1*). The mothers of these hybrids came from a colony of strain C mice in which less than one percent of breeding females developed mammary tumors (*2*), and their fathers were from a colony of strain C_3H mice in which over 90 percent of breeding females developed these tumors (*3*). The fact that the average age at which the hybrids developed tumors was considerably higher than that of inbred strains suggested that if strain C milk contained an agent it must have been either attenuated or present in small amounts. The assumption that strain C mice could propagate an attenuated agent appeared logical because it was known that when they obtained an active agent, they revealed a high incidence of tumors and transferred the agent through successive generations (*3*). Therefore, a number of experiments were started to detect, if possible, the mammary tumor-agent in strain C females.

In one experiment (*1*) hybrid mice known to be very susceptible to the agent were suckled by strain C foster mothers and showed a mammary-tumor incidence of only 8 percent, whereas $C \times C_3H$ hybrids born to and suckled by the same C females showed an incidence of 60 percent. In another experiment (*2*) an effort was made to enhance the activity of the C-strain agent by passage through highly susceptible mice. Agent-free strain C_3H mice (*4*) were foster nursed by C females and then bred brother-to-sister for three generations, but their incidence of tumors was no higher than in nonfostered controls. In another investigation (*5*) further efforts were made to ascertain whether the F_1 hybrids had acquired the agent

[1] Received for publication January 23, 1950.

from their strain C mothers. The F_1 hybrids were bred to procure backcross and successive hybrid generations. As usual the F_1 hybrids showed a high incidence of tumors but their progeny showed much lower incidences. This indicated that if the F_1 hybrids obtained the agent from their C mothers its activity was not enhanced in their descendants. The outcome of these investigations suggested that strain C milk did not contain the mammary tumor-agent.

This paper reports another effort to enhance the activity of the agent in strain C mice, or to lower the resistance of the mice to the agent, by the use of X-radiation. This procedure was employed because of the mutagenic properties of X-radiation and because Lorenz *et al.* (*6*, *7*), reported the production of mammary tumors in mice by prolonged irradiation with daily small dosages of gamma radiation.

The investigations did not produce conclusive evidence that X-radiation influenced the activity of the agent but it confirmed the occurrence of mammary tumors in hybrids derived from C females and C_3H males and the presence of the agent in a small number of the hybrids that developed tumors at an early age. These earlier results were extended by showing that, after mating with strain C_3H males, some strain C females also carried the agent but the presence of the agent in these C females was not correlated with its presence in all their $C \times C_3H$ offspring.

MATERIALS AND METHODS

All animals were raised in this laboratory. They were maintained under similar environmental conditions, given an unlimited supply of tap water, and fed Purina laboratory chow pellets. Those under observation for the appearance of mammary tumors were kept eight to a cage and were examined every other week for palpable tumors.

Histologic sections were prepared from every tumor. The tissues were fixed in Tellyesniczky's fluid (2 parts formol, 20 parts 70 percent alcohol and 1 part acetic acid) and stained with hematoxylin and eosin. A discussion of the morphology of these tumors will be presented later in this paper.

Various procedures were used to test for the presence of the mammary tumor-agent in tumors and tumor-bearing mice. These consisted of the following: (1) Breeding the animals suspected of carrying the agent and observing the resultant female offspring for the appearance of tumors. (2) Inoculation of pieces of tumor or spleen or both from the tumor-bearing mouse into test mice that were susceptible to the agent but were genetically resistant to the growth of the implanted tissues. All implants were placed subcutaneously in the right axilla by means of the usual trocar technique. (3) Administration of tumor extracts to susceptible mice. Tumor extracts were prepared by grinding 1 gram of tumor tissue with sand, and diluting in 5 cc. of distilled water or phosphate buffer (0.1M, pH 8.0). The mixtures were kept at room temperature for approximately 5 minutes until the heavy particles settled. The supernatant

was used to inoculate the test animals, each mouse receiving 0.1 or 0.2 cc. intraperitoneally.

Test animals for the inoculation of tumor or spleen, or tumor extracts, were 7-to-14-day-old female mice of strain C, agent-free strain C_3H mice, or hybrids derived from strain C females and agent-free C_3H males. These test animals were mated when 2 months old and permitted to bear one litter, which was removed soon after birth. They were kept until at least 18 months of age.

Single doses of whole-body X-radiations were administered under the following irradiation conditions: 186 KV (peak), 20 ma., added filtration 0.25 mm. Cu. and 1.06 mm. Al., 50 cm. focus-middle-mouse distance, dose rate 57 r per minute.

EXPERIMENTAL PROCEDURE AND RESULTS

The experiment was started by irradiating 25 litters of strain C female mice. One mouse of each litter was kept as a nonirradiated control; the others were given single doses of 25, 50, 100, or 200 r of X-radiation over the whole body. Members of each litter received different amounts of X-radiation: an ideal litter contained 5 mice; 1 served as control, the others received 25, 50, 100, or 200 r.

The mice in 15 of the litters were 1 to 4 days old and those in 10 litters 12 to 14 months old when they were irradiated. Those in the older age group had given birth to 3 or 4 litters before exposure.[2]

The older mice were bred immediately after exposure and the younger animals when 2 to 3 months of age. All were mated to strain C_3H males, and, when possible, each raised 2 litters of F_1 hybrids. When the litters were weaned the only mice kept were 1 female and 1 male from each litter born to nonirradiated C females and 2 females and 1 male from each litter born to the irradiated mice.[3]

Mice born to strain C females and strain C_3H males were designated $(C \times C_3H)F_1$ hybrids and were obtained to determine whether the milk of their strain C mothers contained the mammary tumor-agent. It was hoped that if exposure of C females to X-radiation activated or enhanced a mammary tumor-agent in them, then the incidence of mammary tumors in their F_1 hybrids, together with the average age at which the tumors arose, would reflect the degree of enhancement or activation of the agent.

When the F_1 hybrids were 2 to 3 months old they were bred to nonirradiated male litter mates and kept continuously with them from 4 to

[2] All mice exposed to X-radiation within 24 hours after birth produced litters, but those exposed when 2 to 4 days old did not become pregnant if they had received more than 25 r. Of the 12-to-14-month old group only 3 produced litters: These had received 25, 50, and 100 r, respectively, and each cast 1 litter only.

[3] After weaning the second litters, all strain C females that were irradiated when less than 2 days old were kept with males for an additional 5 months to ascertain whether exposure to X-radiation influenced their ability to become pregnant. They were kept with males continuously and their litters removed soon after birth. Five that received 25 r produced an average of 5.4 litters; and 5 that received 50 r, an average of 4.6 litters. Of 5 that received 100 r, 3 became pregnant and produced an average of 3.7 litters. Of 7 that received 200 r, only 1 became pregnant and bore but 1 litter.

6 months. Each mouse cast from 4 to 8 litters, and all their offspring were removed soon after birth.

RESULTS IN F_1 HYBRIDS

Results in the F_1 hybrids are summarized in table 1. The table includes the 3 F_1 hybrids born to C mothers that were irradiated when 12 to 14 months old and the 14 F_1 hybrids born to nonirradiated C females of the same age group. These hybrids are included because results in them were similar to those observed in hybrids born to the younger strain C females.

Tumor incidences of 71 and 86 percent in the hybrids born to nonirradiated C mothers approximated the results of a previous experiment (*5*), when similar hybrids which also bore from 4 to 8 litters apiece showed an incidence of 79 percent mammary tumors. Thus, the relatively high incidence of mammary tumors attending the hybridization of low-tumor C females and high-tumor C_3H males was again confirmed.

Tumor data in hybrids born to irradiated strain C females were similar to those born to nonirradiated C females. The only difference was the lower average age at which tumors occurred in offspring born to C females that received 200 r of X-radiation. The appearance of tumors in four 5-to-7-month-old mice of these groups was responsible for this difference because, when these mice are excluded, the average age at which the first-litter mice developed tumors becomes 22 months and that of the second-litter animals becomes 27 months.

Five $C \times C_3H$ hybrids born to four irradiated C females developed tumors when 5 to 7 months of age, and all five carried the mammary tumor-agent. Further experimentation is essential to determine whether the presence of the agent in these mice was the result of irradiating their strain C mothers because, in a previous experiment (*5*), six $C \times C_3H$ hybrids born to nonirradiated C females also contained the agent.

Detection of the agent in the five hybrids was easily accomplished by permitting them to suckle their litters. These mice and six generations of their descendants developed mammary tumors. Furthermore, tumors arose in susceptible mice that were suckled by the descendants of the hybrids.

Despite the failure of previous efforts (*1*, *5*) to detect the agent in older F_1 hybrids after they had developed tumors, further attempts were made in the present experiment. Since extracts of tumors arising in older $C \times C_3H$ hybrids failed to produce tumors in test mice of the earlier studies, a different procedure was used to test for the agent in this experiment. The tumors were transplanted into $C \times C_3H$ hybrids from 3 days to 3 months old; most of the mice were approximately 1 month old when inoculated. All inoculations were made subcutaneously in the right axilla by the usual trocar technique. It was hoped that if tumors arising in the older mice were produced by an attenuated agent, or if they contained a large amount of agent inhibitor, transplantation into young animals

TABLE 1.—*Occurrence of mammary tumors in (C×C3H) F_1 hybrids derived from irradiated and nonirradiated strain C females and nonirradiated strain C3H males*

Dose of X-radiation to C mother	Litter No.	Number of mice	Mice developing tumors / Mice dying without tumors — Age in months																						Total	Tumor incidence	Average age at which tumors occurred	Average age at death without tumor
			5	6	7	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30				
r																										*Percent*	*Months*	*Months*
0	1	24	.....	.....	.....	.....	.....	1/0	.....	1/0	.....	2/1	2/0	1/1	4/1	.....	3/0	1/1	.....	0/2	.....	1/0	1/1	.....	17/7	71	21	23
0	2	14	.....	.....	.....	.....	.....	.....	.....	1/0	.....	.....	.....	1/0	2/0	1/0	4/0	1/0	.....	1/1	1/0	0/1	.....	.....	12/2	86	22	27
25	1	14	.....	.....	.....	.....	.....	.....	.....	1/0	.....	1/0	.....	.....	2/0	1/1	2/1	1/1	1/0	.....	.....	.....	1/0	0/1	10/4	71	22	25
25	2	14	.....	.....	.....	.....	.....	.....	.....	.....	.....	1/1	0/2	1/0	.....	.....	.....	3/1	1/0	2/0	.....	0/1	0/1	.....	8/6	57	24	23
50	1	11	.....	.....	.....	.....	.....	.....	1/0	1/0	.....	.....	.....	1/0	.....	4/0	.....	1/0	1/1	.....	.....	.....	1/0	.....	10/1	91	22	25
50	2	10	.....	.....	.....	1/0	.....	.....	.....	.....	.....	.....	.....	.....	1/2	.....	.....	1/1	.....	1/0	1/1	0/1	.....	.....	5/5	50	22	24
100	1	11	.....	.....	.....	.....	.....	.....	.....	.....	1/0	2/0	.....	1/0	1/0	2/0	1/0	2/0	.....	.....	.....	.....	.....	1/0	11/0	100	22	.....
100	2	11	.....	1/0	.....	.....	.....	.....	.....	1/0	.....	.....	.....	.....	0/1	1/0	1/2	.....	1/0	2/0	.....	1/0	.....	.....	8/3	73	22	22
200	1	12	.....	1/0	1/0	.....	1/0	.....	0/1	.....	1/1	.....	.....	.....	2/0	.....	1/0	.....	1/0	.....	1/0	1/0	.....	.....	10/2	83	19	16
200	2	7	1/0	1/0	.....	.....	.....	.....	.....	.....	.....	.....	0/1	.....	.....	.....	.....	.....	.....	1/0	2/0	0/1	.....	.....	5/2	71	18	24
Total	.....	128	1/0	3/0	1/0	1/0	1/0	1/0	1/1	5/0	2/1	6/2	2/3	5/1	12/4	9/1	12/3	10/4	5/1	7/3	5/1	3/4	3/2	1/1	96/32	75	21	23

would provide a soil for rapid growth, and the agent would become more active or the inhibitor would decrease in amount.

This test involved the use of animals that were genetically susceptible to the growth of implanted tumor tissue, and the resultant tumor was tested for the presence of the agent. Hereafter the test is referred to as transplantation of tumor tissue. It should not be confused with the test described in the section on Materials and Methods, which involved the use of animals genetically resistant to the growth of implanted tumor tissue and was limited, therefore, to the presence or absence of the amount of agent in the small piece of inoculum. Hereafter this test is referred to as inoculation of tumor tissue.

Thirty-seven tumors from hybrids 18 to 24 months of age were transplanted into the young animals. Each tumor was used to inoculate 2 females and 2 males from different litters; each litter contributed 1 male and 1 female.[4]

Of the 37 tumors, 23 produced definite growths within a month after the first transplantation, 10 were palpable between the first and second months; one did not appear until 3.5 months, and one until 7 months following transplantation. Two did not grow within a 3.5-month period that the mice were kept under observation. Nineteen of the most rapidly growing tumors and 2 that were palpable between the first and second months were tested for the presence of the agent. Of these, 15 were in the first transplant generation, 3 in the second, 2 in the third, and 1 in the fourth. Seventeen were tested by means of tumor extracts, and 4 by the inoculation of pieces of tumors and spleens of tumor-bearing animals. None of the test mice developed a mammary tumor.

RESULTS IN STRAIN C FEMALES

A total of 81 strain C females was used. Fifty-nine belonged to the 15 litters irradiated when 1 to 4 days of age, and, of these, 15 were nonirradiated controls: doses of 25, 50, 100, and 200 r of X-radiation were given to 10, 10, 10, and 14 mice, respectively. Twenty-two belonged to the 10 litters irradiated when 12 to 14 months old and, of these, 10 were nonirradiated controls: doses of 25, 50, 100, and 200 r of X-radiation were given to 4 series of 3 mice each.

Six of the strain C females developed mammary tumors. They belonged to 5 litters, all of which included mice irradiated when less than 2 days of age along with their nonirradiated control litter mates. Two were nonirradiated controls, 2 received 50 r, 1 received 100 r, and 1 received 200 r of X-radiation. The occurrence of tumors in 6 of the 81 strain C mice of this experiment was unusual for, thus far, only 6 of 650 strain C colony breeding females have developed mammary tumors (*2*).

The 6 tumor-bearing C females of this experiment are included in table 2, in which the occurrence of tumors in their offspring and in the

[4] Foulds (*8, 9*) transplanted mammary tumors arising in hybrids derived from C57 black females and R3 males and found that some of the transplanted tumors grew better in females. In the transplantation of C×C_3H tumors reported here only 1 showed a similar sex difference in transplantability; the others grew equally well in both sexes.

offspring of 2 other C females is summarized. The table also includes the 5 hybrids that developed tumors at an early age and contained the tumor agent.

TABLE 2.—*Family histories in which either the strain C mothers developed mammary tumors or the F_1 hybrids developed mammary tumors at an early age* [1]

Strain C mothers				C × C_3H F_1 hybrid offspring				
				First litter		Second litter		
Mouse No.	Dose of X-radiation	Age at which tumor occurred	Age at death without tumor	Mouse No.	Age at which tumor occurred	Mouse No.	Age at which tumor occurred	Age at death without tumor
	r	*Months*	*Months*		*Months*		*Months*	*Months*
21+	100	21		132	20	193+	6	
34	0	19		97	18	178	23	
39	0	20		155	28	209		26
				156−	20			
45	50	19		144−	22	200	27	
						201	26	
47−	200		9	139+	6	189	26	
						190+	6	
55+	200		8	134	23	198−	27	
				135	21	199+	5	
58+	50	9		112	16	185	21	
				113	24	186		21
61+	200	10		154+	7			

[1] A sign after the number of a mouse denotes it was tested for the presence of the mammary tumor-agent. A + sign denotes the presence of the agent and a − sign its absence.

A detailed account of each strain C mouse of table 2 and its family is presented below. The accounts include a record of tests for the presence of the mammary tumor-agent in the C females and their litter mates, together with pertinent information concerning the occurrence of tumors in the offspring of the litter mates. It should be kept in mind that the primary purpose of the experiment was to ascertain whether the agent was present in the C females and their offspring. Hence, the words "test" and "tested" in the following descriptions denote efforts to detect the presence of the mammary tumor-agent in tumors or tumor-bearing mice by administration of tissues or tissue extracts to susceptible mice. The occurrence of tumors in the C×C_3H hybrid offspring of C females is also presented as evidence of the presence or absence of the agent. A hybrid developing a tumor before a year of age may have contained the agent; those developing tumors when over 15 months of age were, according to present evidence, free of the agent.

Mouse No. 21 had received 100 r of X-radiation and was tested by the inoculation of 4 animals with pieces of her spleen; all developed tumors at an average age of 10 months. Thus, C female No. 21 was shown to contain the agent. She produced 2 hybrids, of which one developed a tumor when 6 months old and contained the agent; the other developed her tumor at the age of 20 months and was not tested. Mouse No. 21 had 4 litter mates of which 2 were tested by inoculation of their spleens into 11 animals; all died free of tumor at a mean age of 17 months. The

4 litter mates of No. 21 produced 7 hybrids, of which 3 developed tumors when 12, 19, and 25 months old, and 3 died tumor free at an average age of 23 months. Pieces of tumor and spleen from the 12-month-old hybrid were used to inoculate 10 test animals, all of which died without tumors at 19 months of age.

C female No. 34 was not exposed to X-radiation and was not tested. Her 2 offspring developed tumors when 18 and 23 months old and were not tested. She had one litter mate that received 200 r of X-radiation and died without tumor when 9 months old. Her 2 progeny developed tumors when 25 and 28 months of age and were not tested.

Mouse No. 39 was not exposed to X-radiation and was not tested. Two of her 3 offspring developed tumors when 20 and 28 months old, and 1 died without tumor at the age of 26 months. The tumor from the 20-month-old hybrid was transplanted and an extract was administered to 5 mice, none of which developed a tumor. Two litter mates of No. 39 received 50 and 200 r of X-radiation, respectively: both died free of tumor when 14 and 16 months old and were not tested. They produced 6 hybrids of which 3 developed tumors at a mean age of 21 months and 3 died tumor free at the same mean age. Two tumors were transplanted and tested; one by the inoculation of tumor and spleen and the other by administration of tumor extract. All test animals died tumor free at the age of 18 months.

Mice Nos. 45 and 47 were litter mates. No. 45 was given 50 r and was not tested. All 3 of her progeny developed tumors when 22, 26, and 27 months old. The tumor appearing in the 22-month-old hybrid was transplanted and grew rapidly. An extract was administered to 4 animals that died tumor free at the age of 16 months.

No. 47 received 200 r and died without tumor when 9 months old. Her spleen was tested by inoculation into 4 mice: all died tumor free when 18 months old. However, 2 offspring of No. 47 developed tumors when only 6 months old and both contained the agent. Her remaining offspring, No. 189, developed a tumor at the age of 26 months but was not tested.

Three litter mates of mice Nos. 45 and 47 died without tumor at a mean age of 20 months. The spleen of 1 that had received 25 r of X-radiation was tested by inoculation into 5 mice; all died tumor free at a mean age of 14 months. The 3 litter mates raised 6 hybrids; 4 developed tumors at the average age of 20 months, and 2 died tumor free when 21 and 23 months old. Three of the tumors were transplanted and, after 1 or 2 passages, extracts were tested by injection into 28 mice. These li[illegible]d to an average age of 18 months and died free of tumor.

Strain C female No. 55 was given 200 r and died tumor free when 8 months old. Her spleen was transplanted into 6 animals: 4 developed tumors when 7 to 10 months old, and 2 died without tumor at the ages of 15 and 19 months. This indicated that C female No. 55 contained the agent. All 4 of her progeny developed tumors. One, No. 199, was 5 months of age and possessed the agent. The litter mate of No. 199

developed her tumor when 27 months old. She was tested for the agent by the implantation of pieces of spleen into 5 animals. All died tumor free at 18 months of age. The other 2 progeny of No. 55 developed tumors when 21 and 23 months old and were not tested.

Mouse No. 55 had 3 litter mates that died tumor free at a mean age of 16 months; none was tested. They raised 9 hybrids, of which 5 developed tumors at an average age of 19 months and 4 died without tumors at a mean age of 26 months. One tumor was transplanted and the resultant growth tested by administration of an extract. The test mice died tumor free at 18 months of age.

The last litter represented in table 2 contained mice Nos. 58 and 61. No. 58 was given 50 r and was tested by implanting pieces of her spleen into 5 animals; all developed tumors at an average age of 8 months. Of 4 offspring born to No. 58, 3 developed tumors when 16 to 24 months old and 1 died tumor free at the age of 21 months; none was tested.

No. 61 received a dose of 200 r and was tested by the inoculation of pieces of her spleen into 8 mice, all of which developed tumors at an average age of 8 months. Table 2 shows that her only offspring, No. 154, developed a tumor when 7 months old and was, therefore, one of the hybrids that carried the agent.

Five litter mates of Nos. 58 and 61 died without tumors at an average age of 18 months and were not tested. They produced 15 hybrids, of which 13 developed tumors at an average age of 22 months. Four tumors were transplanted into young mice and the resultant growths tested by administration of tumor extracts. Test mice lived to be 18 months old and none developed a tumor.

The findings in these 6 litters of strain C females are summarized below.

Litter No. 1 contained irradiated female No. 21 that developed a tumor and contained the agent. She had 2 offspring, 1 carried the agent and 1 was not tested. Of her 4 strain C litter mates 2 did not contain the agent and 2 were not tested. Of 7 offspring born to the litter mates, 3 developed tumors: one tumor was tested with negative results.

Litter No. 2 contained nonirradiated female No. 34 who developed a tumor and was not tested. Her offspring developed tumors and were not tested. A litter mate of No. 34 had 2 progeny, both of which developed tumors. Neither she nor her progeny was tested.

Litter No. 3 contained nonirradiated female No. 39 who developed a tumor and was not tested. Of her 3 offspring, 2 developed tumors; 1 tumor was tested, with negative results. Two litter mates of No. 39 died tumor free and were not tested. They produced 6 progeny, of which 3 developed tumors: 2 tumors were tested with negative results.

Litter No. 4 contained irradiated females Nos. 45 and 47. Mouse No. 45 developed a tumor and was not tested. All 3 of her progeny developed tumors: 1 tumor was tested with negative results.

Mouse No. 47 did not develop a tumor and she did not carry the agent. All 3 of her progeny developed tumors: 2 carried the agent and the other was not tested.

Nos. 45 and 47 had 3 litter mates: 1 was tested with negative results. They raised 6 hybrids of which 4 developed tumors: 3 tumors were tested with negative results.

Litter No. 5 contained irradiated female No. 55 that did not develop a tumor but carried the agent. All 4 of her progeny developed tumors: 1 contained the agent and 1 was tested with negative results. Mouse No. 55 had 3 litter mates that died free of tumor and none was tested. They produced 9 hybrids of which 5 developed tumors: 1 tumor was tested with negative results.

Litter No. 6 contained irradiated females Nos. 58 and 61, both of which developed tumors and carried the agent. Of 4 progeny born to No. 58, 3 developed tumors; none was tested. The only female born to No. 61 contained the agent.

Five litter mates of Nos. 58 and 61 were not tested. Of their 15 offspring, 13 developed tumors; 4 tumors were tested with negative results.

Thus, tests for the presence of the mammary tumor-agent in families represented by the 8 strain C females of table 2 produced the following results:

(1) Three of the 6 tumor-bearing C females were tested, and the agent was present in all of them.

(2) The 4 strain C mothers of the 5 hybrids that contained the agent were tested. The agent was detected in 3 but not found in the other.

(3) The 8 C females produced a total of 22 hybrids. Five of these developed tumors at an early age and carried the agent. Only 3 of the remaining 17 hybrids were tested, and the agent was not detected in them. Two of the tested hybrids (Nos. 144 and 156) were born to C females Nos. 39 and 45, both of which developed tumors but were not tested. The other tested hybrid, No. 198, was of interest because her mother as well as her litter mate contained the agent.

(4) Of 18 litter mates of the 8 C females only 3 were tested, and the agent was not found.

(5) Of 45 $C \times C_3H$ offspring born to the 18 litter mates, 30 developed tumors. Ten tumors were tested after transplantation into young mice, and all tests were negative.

Thus, of the 8 strain C females listed in table 2, 5 (Nos. 21, 47, 55, 58, 61) were tested, and the agent was found in 4 (Nos. 21, 55, 58, 61). These were the first strain C mice of this series of investigations that were shown to carry the agent. Three (Nos. 21, 55, 61) were mothers of hybrids that carried the agent and 1 (No. 58) did not produce an agent-bearing hybrid.

One C female (No. 47) in which the agent was not detected produced 2 agent-bearing hybrids. Furthermore, the foregoing detailed analysis of the families to which the agent-carrying C females belonged suggests that their litter mates did not contain the agent. In addition to the C females included in the above analysis, 7 more, representing 6 other litters, were tested for the agent by transplanting their splenic tissues. All these C females had received 100 or 200 r of X-radiation, and none

had produced offspring. A total of 27 mice received the splenic transplants; all died free of tumor at a mean age of 17 months.

Further analysis of the eight C females of table 2 was made to ascertain their relationship to the colony of C mice maintained in this laboratory. Nos. 58 and 61 were litter mates, and their mother in the colony was a litter mate to the mother of No. 21. Both colony mice died without tumor when 17 and 25 months old. They raised 8 offspring in the colony, and these mice (sisters to Nos. 21, 58, and 61) all died tumor free at an average age of 18 months. The colony mother of litter mates Nos. 45 and 47 died free of tumor when 23 months old. She had 19 descendants in the colony, which lived to a mean age of 15 months; none developed a mammary tumor. The mothers of Nos. 34, 39, and 55 were not colony members but were first-generation offspring of colony females and lived to the ages of 22 and 25 months without developing mammary tumors. Furthermore, their mothers must have died without tumors because none of the breeding females of their generation in the colony developed a mammary tumor.

The data presented above are evidence that the strain C females of table 2 did not acquire the agent from their colony mothers. Indeed, all the available information emphasizes the random distribution of the C females that carried the tumor agent.

DISCUSSION

The experiment reported here was one of a series of investigations which had, as its primary objective, the demonstration of the mammary tumor-agent in low-mammary-tumor-strain C females. In this experiment an attempt was made to enhance the activity of the agent or to decrease the resistance of the animals to the agent by exposing new-born strain C females to whole-body X-radiation. Relatively small doses (25 to 200 r) of X-radiation were given because it was essential to procure litters from the irradiated females. The C females were bred to strain C_3H males because F_1 hybrids derived from these strains were known to show a high incidence of mammary tumors at a late average age. It was expected that if irradiation of the strain C females increased the activity or amount of agent in them, then this increase would be reflected in a higher incidence of mammary tumors at an earlier average age in their F_1 hybrid offspring.

The over-all results indicated that the procedure did not influence the activity of the agent, because the incidence of mammary tumors in offspring of nonirradiated C females was the same as in those derived from irradiated animals.

The findings confirmed those of other experiments (*1*, *5*) in which hybrids born to low-mammary-tumor-strain C females and high-mammary-tumor-strain C_3H males revealed a high incidence of tumors. The majority of tumors occurred in older mice, and efforts to reveal the agent in these tumors have, thus far, met with failure. In this experiment the tumors of older mice were transplanted into young $C \times C_3H$ F_1 hybrids on the assumption that growth in young animals may lead to an increase

in the activity of the agent or to the loss of an agent inhibitor. The procedure failed to detect the presence of the agent.

In the present experiment most of the strain C females raised 2 litters. Examination of the results showed that, as in the earlier experiments, the tendency toward tumor development in the older hybrids was not limited to certain C mothers or C_3H fathers. Hybrids belonging to first and second litters showed similar incidences, and those born to nonirradiated C females developed as many tumors at approximately the same average age as those born to C females that had received 25, 50, 100, or 200 r of X-radiation. Thus, the experiment did not contribute any new information concerning the genesis of tumors in older $C \times C_3H$ hybrids.

Five hybrids born to C females that had received 100 or 200 r of X-radiation developed tumors when only 5 to 7 months of age, and these mice contained the agent. This finding, however, was similar to that of earlier experiments (*5*) in which the strain C females were not exposed to X-radiation. The sudden appearance of the agent in offspring of irradiated females may have been fortuitous or it may have been the result of exposure to X-radiation increasing or accelerating the occurrence of a biologic reaction. This problem is receiving further attention.

Data in table 2 show that in the present experiment the 5 young tumor-bearing hybrids belonged to 4 litters, and also reveal the random distribution of these mice in respect to litter sequence and litter mates. Two belonged to first litters and 3 to second litters. Two (Nos. 139 and 190) were sisters; No. 139 was the only member of the first litter, and No. 190 belonged to the second litter. Her litter mate, No. 189, did not develop her tumor until she was 26 months old. Another, No. 199, belonged to a second litter and developed her tumor at the age of 5 months. Her litter mate was 27 months old and her sisters of the first litter were 21 and 23 months old when they developed tumors.

In the earlier experiment (*5*) tumors arose in 6 young $C \times C_3H$ F_1 hybrids. Four were the only female members of one litter and 2 belonged to a litter containing 3 females. The remaining mouse of the latter litter died without tumor when 25 months old, and her offspring also failed to develop tumors. Hence, 5 mice of the present experiment presented additional evidence supporting the random distribution of mice in which the agent appeared suddenly.

The 4 tumor-bearing litter mates of the previous investigation (*5*) were bred to brothers and 5 generations of descendants procured by brother-to-sister matings. Descendants of 3 revealed high incidences of tumors in every generation whereas the descendants of 1 contained a number of mice that failed to develop tumors. This suggested the inactivation or disappearance of the agent in progeny of a $C \times C_3H$ F_1 hybrid in which it had appeared suddenly. Because of this observation 6 generations of descendants were procured from each of the 5 agent-containing hybrids of this experiment to explore further the possibility of the agent's inactivation or disappearance. The details of this part of the study are not

presented in this report because all the progeny showed high incidences of tumors.

Definite extension of earlier findings was attained by testing the strain C mothers of tumor-bearing hybrids for the presence of the mammary tumor-agent. In the preceding experiments the mothers of the litters in which the agent was found were not tested for the agent because one died before tumors appeared in her offspring, and the other died before a diagnosis was made of the first tumor in her offspring.

In this experiment the 4 C mothers of hybrids that carried the agent were available. Two, Nos. 21 and 61 (table 2), developed mammary tumors and 2, Nos. 47 and 55, died without tumors. All were tested for the presence of the agent by implanting pieces of their spleens into susceptible mice. The agent was detected in 3, Nos. 21, 55, and 61, and was not detected in 1, No. 47.

Four other strain C females, Nos. 34, 39, 45, and 58 (table 2), also developed mammary tumors but only 1, No. 58, was tested for the agent. She was a sister of No. 61, which developed a tumor when 10 months old, contained the agent, and produced 1 offspring that carried the agent. No. 58 developed her tumor when 9 months old, contained the agent, but presumably none of her 4 offspring harbored the agent. Furthermore, 5 strain C litter mates of Nos. 58 and 61 died tumor free at a mean age of 18 months, and none of their 15 offspring developed a tumor at an early age.

Table 2 also contains other equivocal findings. For example, C female No. 55 possessed the agent but only 1 of her 4 hybrid offspring did so, whereas the agent was not found in C female No. 47 but it was present in 2 of her 3 offspring.

There is no satisfactory explanation for these results. In a previous paper (*5*) four possibilities were suggested to explain how the agent gained access to the $C \times C_3H$ F_1 hybrids. These were (1) through contagion, since the agent is transmissible and is found in the blood; (2) by way of the milk of their strain C mothers, which may harbor a weak or masked agent and which became activated in a few hybrids; (3) from their strain C_3H fathers, which are known to carry the agent; and (4) by appearing spontaneously in the hybrids.

The experiment reported here produced discrepancies between the presence of the agent in C females and the occurrence of tumors in their offspring, which complicate any of the above possibilities. If C female No. 58 of table 2 acquired sufficient agent to develop a tumor at 9 months of age, then why did none of her 4 offspring develop a tumor in early life and carry the agent? She may have acquired the agent after raising her litters, or she may have carried an active agent and transmitted a very small amount to her offspring by way of her milk. But this interpretation would not explain the results in litters born to C females Nos. 21 and 55. Both these mice possessed the agent and raised 1 agent-containing hybrid but they also raised hybrids which, according to all present evidence, did not possess the agent. Hence, if the agent-bearing

hybrids acquired the agent from their mothers' milk, then they were the only ones to do so despite the presence of sister litter mates. Female No. 47 presented another paradox, for apparently she and one of her offspring did not contain the agent, but her 2 other offspring did.

The possibility that all the C females obtained the agent through contamination appears improbable unless they became infected after their litters were born. This postulation fails to explain the presence of the agent in certain offspring of Nos. 21 and 55 unless they also were infected through contamination. If the C females and a few of their offspring acquired the agent from contaminated excreta or by way of insect transmission then, thus far, they are the only mice in this laboratory to do so, despite the numerous opportunities for similar infection in other equally susceptible mice.

The possibility that exposure to X-radiation activated a latent agent in the C females is receiving further consideration because in this experiment the agent was detected in only those C females which had been irradiated. This possibility appears remote because a number of other irradiated C females were tested for the agent with negative results and all offspring of the agent-containing C females did not carry the agent. However, Lorenz *et al.* (*6*, *7*), reported the production of mammary carcinomas and mammary sarcomas in mice that were exposed to long-continued irradiation. They used 2-to-3-month-old F_1 hybrids which were presumably deficient in the agent because they were derived from strain L females and strain A males, and they set forth the hypotheses that the hybrids may have carried a weak agent or inhibitor, and that daily small dosages of gamma radiation over long periods of time activated the agent or destroyed the inhibitor.

The possibility of the agent arising spontaneously within an occasional mouse offers an explanation for all the conflicting results encountered in this experiment but, as stated before (*5*), such evidence is attainable only by the elimination of all other possibilities.

The possibility that the C females and hybrids may have obtained the agent from the C_3H males deserves consideration because it offers an explanation of the equivocal results on the basis of knowledge of transmissible diseases and the mammary tumor-agent. It was shown that the agent is in the seminal vesicles of strain C_3H males (*4*). This indicates that the agent was transferred to the C females and the hybrids may have acquired their C_3H fathers' agent while *in utero*. All offspring would not acquire their fathers' agent if it were carried in a relatively small number of spermatozoa or entered the ovum at the time of fertilization.

Infection of only a few embryos could account for the random distribution of agent-bearing hybrids, and these infected embryos may or may not have infected their strain C mothers. In an earlier experiment (*5*) an attempt was made to ascertain whether C females acquired infection through contact with C_3H males by procuring offspring from C females bred to C_3H males and then to strain C males. A low incidence of tumors in the C×C offspring indicated that the C females were not

infected by mating with C_3H males. This result, together with the findings presented herein, indicates that only a small number of C females acquired the agent from their C_3H mates. Hummel, Little, and Eddy (*10*) have reported that the agent is destroyed or inhibited by the placenta, and this may explain why the agent may not gain access to the mother while the infected young are *in utero*.

Infection of C female No. 58 by way of an infected male embryo would explain the absence of agent-bearing female offspring.[5] Infection of C females Nos. 21 and 55 through embryos of their second litters could explain the absence of the agent in their first-litter hybrids. Mouse No. 47 could have given birth to infected young in both litters but she may not have obtained the agent from either.

The outcome of this investigation did not provide a solution to the major problem of the etiology of mammary tumors in $C \times C_3H$ F_1 hybrids, namely, whether or not the mammary tumor-agent was involved in the occurrence of all mammary tumors in these animals or whether it was implicated only in those tumors arising in young hybrids. It did, however, show that after mating with strain C_3H males, some strain C females contained the agent which, apparently, they did not transmit to all their $C \times C_3H$ offspring.

Foulds (*11*) found that some F_1 hybrids born to low-mammary-tumor-strain C57 black mothers and high-mammary-tumor-strain R3 fathers developed mammary tumors at a relatively early age, and these mice carried and transmitted the agent. In the discussion of his findings, Foulds stated that they "indicated a transmission of tumor agent by males of high-tumor strains to their hybrid offspring."

A series of experiments now in progress was designed to elucidate further the role of high-mammary-tumor-strain males in the occurrence of mammary tumors in their F_1 hybrids.

HISTOLOGIC APPEARANCE OF THE TUMORS

In a recent paper (*12*) some of the difficulties in the classification of mammary tumors in mice were discussed. It was pointed out that no rigid classification was possible because of the variations from area to area in many tumors and the gradual merging of one type into another. Where only a few tumors are to be diagnosed, the inclusive term "mammary tumor" is adequate. On the other hand, where large numbers of tumors from a uniform animal source are available for study, an attempt at sorting them, using particular morphologic criteria, may reveal a preponderance of tumors with some unusual feature. When collections of mammary tumors from large groups of mice differing in known factors have been compared, significant differences in the morphology of the tumors have been found. This is particularly true when a comparison of animals with and without the tumor agent has been made.

For comparison with the tumors in $C \times C_3H$ hybrids of the present study, a group of 86 tumors derived from the mammary gland and arising

[5] Other female offspring may have been infected, since only 2 females and 1 male were kept from each litter of irradiated C females.

in strain C mice that were known to possess the tumor agent, were available. The tumor-bearing mice were from 6 to 12 months of age. These tumors were sorted, and it was found that they could be roughly separated into the following two groups:

1. Adenocarcinoma (type A), with acinar structure predominating. In this group were placed tumors in which one half or more of the area of the tumor included in the section was composed of a fine glandular or tubular structure suggesting an origin from the mammary acini. Forty-five of the 86 tumors, or 52+ percent, were placed here.

2. Adenocarcinoma (type B), with very variable patterns, and with less than half to no areas in which an acinar structure was reproduced. As is often true in sorting mammary tumors in mice, there was no absolute demarcation between some of these tumors and those of type A, and the amount of acinar reproduction was selected as a criterion. Some of these type B tumors showed predominantly solid groups of epithelial cells, others showed single layers of cuboidal cells lining cystic spaces or growing as papillary projections into cysts. Particularly frequent in these strain C mice were tumors composed of cords of cells, separated by an abundant, collagenous connective tissue. This stromal development in fact helped to distinguish these tumors from the type A tumor, in which the stroma was usually scanty and delicate. It should be stated again that there is no sharp line of division between tumors of these groups; however, the type B tumors showed a wider range of variation in the character and arrangement of the neoplastic epithelial cells and the stroma. Forty tumors, or 46+ percent were placed here. In comparison with other strains of mice with the tumor agent examined at the National Cancer Institute (C_3H and dba), it appears that strain C with the tumor agent has a relatively high proportion of mammary tumors in which an acinar structure does not predominate.

All these 86 strain C tumors except 1 could be sorted into these 2 groups. This exception revealed many squamous-cell elements in section, and it was diagnosed as adenoacanthoma.

With these tumors of strain C mice, with the tumor agent as a background, the 101 tumors from the 96 tumor-bearing $C \times C_3H$ hybrids of the present experiment were examined. Five mice had 2 tumors each, thus accounting for the greater number of tumors than of mice. These multiple tumors were not always of the same histologic type, which shows that the pattern of the individual tumors is not directly dependent upon the host animal. This fact also emphasizes the caution that must be practiced in ascribing any great significance to the morphology of an individual tumor.

The tumors were sorted into four groups according to their microscopic morphology and are summarized in table 3. The groups were as follows:

1. Adenocarcinoma (type A), with acinar structure predominating (fig. 1). Thirty-nine, or 38 percent, of the tumors were placed here. These tumors have been designated as the "typical" or acinar form of mammary tumors in earlier publications (*1*, *2*, *4*, *5*).

TABLE 3.—*Occurrence of mammary tumors in (C×C₃H) F₁ hybrids according to microscopic appearance*

Tumor type	Age in months																						Total
	5	6	7	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	
Type A adenocarcinoma	1	3	1	1	----	1	----	3	----	4	1	1	5	4	----	2	2	5	2	1	1	1	39
Type B adenocarcinoma	----	----	----	----	1	----	1	1	2	2	1	3	5	2	12	5	2	2	3	1	2	----	45
Type C adenocarcinoma	----	----	----	----	----	----	----	----	----	----	----	----	1	1	----	3	1	----	1	1	1	----	9
Adenoacanthoma	----	----	----	----	----	----	----	1	----	----	----	1	1	2	----	----	----	1	----	1	1	----	8
Total	1	3	1	1	1	1	1	5	2	6	2	5	12	9	12	10	5	8	6	4	5	1	101

2. Adenocarcinoma (type B), with variable pattern (figs. 2, 3, 4). Forty-five, or 44 percent, of the tumors were placed here. Most of these tumors closely resembled the type B tumors of the strain C mice with the agent, but others were remarkable in having unusually large cells, much above the size seen in acinar tumors (fig. 2). It is interesting that Haaland (*13*) called attention to the large cell size in certain mammary tumors examined by him, and he expressed the opinion that these tumors may have originated from the ducts. His descriptions of these tumors closely resemble the large cell tumors which we have placed in this second group of adenocarcinomas. Haaland wrote that when the structure of the duct was reproduced, large lumina were formed, often with papillary excrescences, and the cells were often larger and distinctly columnar. Although it can be supposed that certain mammary tumors in mice may originate in the duct, as do the majority in human beings, it is questionable whether a reliable separation regarding ductal or alveolar origin can be made in the mouse.

This group of tumors, which in this paper are designated adenocarcinoma type B, was not separately classified in previous publications on mammary tumors arising in $C \times C_3H$ hybrids because both this form and the acinar type (adenocarcinoma, type A) were grouped together as type I tumors. In this way they were separated from a distinctive form of tumor which occurred with remarkable frequency, and which was catalogued as type II. In the present publication these peculiar, atypical type II tumors are listed as adenocarcinoma, type C, and discussed below.

3. Adenocarcinoma (type C), composed of many epithelial-lined cysts. The epithelial lining of the cysts in these tumors was closely invested by a layer of spindle cells, and it was this character that especially distinguished this tumor from some tumors of type B. In a previous publication (*4*) the question was raised whether these type C tumors were malignant tumors or precancerous lesions. Attention was called to the similarity of these tumors to the changes Haaland (*13*) described as occurring in the mammary glands of old female mice in which areas of epithelial hyperplasia and cystic dilatations were outstanding, but it was noted that the lesions developing in the hybrid mice, in contrast to Haaland's lesions, were of considerable size. These tumors were composed entirely of minute cystic spaces (figs. 5*A* and 5*B*; 6*A* and 6*B*) and the pattern was so characteristic that the tumors were easily separable from other mammary tumor forms. We have never seen a tumor composed entirely of this pattern except in old hybrid mice. Although 2 tumors in strain C mice with the tumor agent showed this pattern in small, usually marginal, areas, the largest part of the section was of a different structure. It is suggested that in younger animals, this pattern is soon overgrown and obscured by other patterns. The youngest animal showing a tumor of this type was 18 months old, and most of them have been found in mice over 22 months in age. This tumor form may be the closest representation to be found in mice of a pure duct origin, and the fact that it arises only in old mice in which acinar tissue has atrophied

may be significant. Nine tumors, or 9 percent, of the present series of tumors in C×C_3H hybrids were placed in this group.

4. Adenoacanthoma, a tumor showing large areas composed of squamous-cell elements (fig. 7). Eight tumors, or 8 percent, were put in this group.

In a previous paper (*12*), this type of tumor was reported to be unusually frequent in methylcholanthrene-painted strain dba mice which were free of the tumor agent. It also appears to be increased in C_3H mice without the agent (*14*).

SPECIAL FEATURES

One interesting tumor was found in a 26-month-old hybrid mouse (No. 201, table 4) which could not be placed in any of the foregoing groups, and it was not included in table 3. It was composed of large cells, some of giant size, which were often detached (fig. 8*A* and 8*B*). Many of the cell nuclei contained eosinophilic inclusions. In the previous report (*12*) on methylcholanthrene-painted dba mice without the tumor agent, a tumor having areas made up of similar detached cells was photographed, but in the present tumor almost the entire cross section showed this cell type, with only a few areas suggesting mammary epithelial tissue.

Another tumor, seen in a strain C female (No. 39, table 4) of the present experiment, was of unusual interest. It was of the "molluscoid" type first described by Haaland and grouped with the adenoacanthomas. This is a rare tumor form which develops a definite pattern, with a central area more or less keratinized surrounded by radiating tubular structures "like the spokes of a wheel" which connect with peripheral glandular formations. In the tumor illustrated here, these "spokes" are seen to infiltrate muscle while still maintaining the tumor pattern (fig. 9). That infiltrative growth should maintain any structural relation to the entire tumor is remarkable.

Two adenocarcinomas (1 among the strain C with the tumor agent, and 1 among the C×C_3H hybrids) were found in which the epithelial cells were vacuolated, probably from secretory activity (fig. 10). No correlation with any condition in the host could be made. Such evidence of secretion in mammary tumors has been discussed by Bonser (*16*).

In many of the tumor sections from the old hybrid mice, a considerable lymphocytic and plasma-cell infiltration was observed in the tissues at the margin of the tumor (figs. 7, 11). Such infiltrations have been described in the mammary glands of old female mice (*13*), but they are seldom seen in young mice with the agent developing mammary tumors at a younger age. Such reactions, therefore, can be correlated to the age and preceding condition of the host tissue rather than to tumor growth.

Careful analyses were made of the tumors arising in the C×C_3H hybrids to ascertain if any correlation existed between the type of tumor and its location, size, rate of growth, dose of X-radiation to the C strain mother of the hybrid, and litter sequence of the tumor-bearing mice, as well as the age of the animals in which they arose. The only correlation

of any significance was between the type of tumor and the age of the mouse. As shown in table 3, 24 tumors appeared in mice 5 to 19 months of age and of these 15, or 63 percent, were type A adenocarcinomas, and 8, or 33 percent, were type B adenocarcinomas, whereas of 77 tumors arising in mice 20 to 30 months old, 24, or 31 percent, were type A, and 37, or 48 percent, were type B. Obviously type A adenocarcinoma, the typical acinar tumor seen so frequently in high-tumor strains of mice, predominated in the younger tumor-bearing hybrids but in the older hybrids the other three types predominated.

There was no relationship between the types of tumors encountered in litter mate C×C_3H hybrids, or between the tumors which appeared in the strain C females and their hybrid offspring. To illustrate this random distribution of tumor types, the diagnoses of tumors arising in the C females and their hybrid offspring of table 2 are presented in table 4. Attention is directed to the tumors occurring in the 3 strain C females shown to possess the agent: 1 was an adenoacanthoma in a 21-month-old mouse, 1 a type A adenocarcinoma in a 9-month-old mouse, and 1 a type B adenocarcinoma in a 10-month-old animal. It is also seen that all 5 hybrids which carried the agent and developed tumors when 5 to 7 months of age had type A adenocarcinoma. Two of the hybrids, Nos. 155 and 201, had 2 tumors each and 1 of the tumors of No. 201 was the previously described tumor which was composed of large cells, often detached (fig. 8*A* and 8*B*).

TABLE 4.—*Microscopic diagnoses of mammary tumors occurring in mice of table 2* [1]

Strain C mothers				C×C_3H F_1 hybrid offspring		
Mouse No.	Dose of X-radiation	Tumor age	Diagnosis	Mouse No.	Tumor age	Diagnosis
	r	*Months*			*Months*	
21+	100	21	Adenoacanthoma	132	20	Adenoacanthoma.
				193+	6	Type A adenocarcinoma.
34	0	19	Type A adenocarcinoma.	97	18	Type B adenocarcinoma.
				178	23	Type B adenocarcinoma.
39	0	20	Adenoacanthoma (see text).	155	28	Type A adenocarcinoma.
				155	28	Type C adenocarcinoma.
				156−	20	Type B adenocarcinoma.
45	50	19	Type A adenocarcinoma.	144−	22	Type A adenocarcinoma.
				200	27	Type A adenocarcinoma.
				201	26	Type A adenocarcinoma.
				201	26	Large cell (see text).
47 [2]	200			139+	6	Type A adenocarcinoma.
				189	26	Type A adenocarcinoma.
				190+	6	Type A adenocarcinoma.
55+ [3]	200			134	23	Type B adenocarcinoma.
				135	21	Type C adenocarcinoma.
				198−	27	Type B adenocarcinoma.
				199+	5	Type A adenocarcinoma.
58+	50	9	Type A adenocarcinoma.	112	16	Type A adenocarcinoma.
				113	24	Type B adenocarcinoma.
				185	21	Type A adenocarcinoma.
61+	200	10	Type B adenocarcinoma.	154+	7	Type A adenocarcinoma.

[1] A sign after the number of a mouse denotes it was tested for the presence of the mammary tumor agent. A + sign denotes the presence of the agent and a − sign its absence.
[2] Died tumor free when 9 months old.
[3] Died tumor free when 8 months old.

Particular attention was given to the tumors of $C \times C_3H$ hybrids that had been transplanted into young mice of the same hybrid derivation. Thirty-seven such tumors had been transplanted, of which 34 first, second, and third transplant generations were available, and these were compared with the original tumors.

Of 10 type A adenocarcinomas, 8 were similar to the originals and 2 resembled type B adenocarcinomas. Of 20 type B adenocarcinomas, one tumor resembled a type A and one had large areas resembling a type C adenocarcinoma, while other areas showed an acinar structure and keratinization. Thus, transplants of the more common tumor types were similar to the original tumors. This is consistent with preceding reports on transplanted mammary tumors (*17*).

Transplants of the adenoacanthomas and type C adenocarcinomas were less consistent in reproducing the original growths. Of 4 transplanted adenoacanthomas, 1 reproduced the primary tumor type, 1 was diagnosed as a type B adenocarcinoma, 1 did not grow until 7 months after inoculation and the resultant tumor was not fixed, and 1 failed to grow within a 3.5-month observation period.

Type C adenocarcinomas also produced a wide range of growth response. Only 3 of these tumors were transplanted. One required between 1 and 2 months to become palpable and developed a pattern of intracystic epithelial proliferation with considerable intervening stroma similar to the majority of type B adenocarcinomas. Another grew slowly and attained a size of 10 by 7 by 6 mm. within 2.5 months. The section of this transplanted tumor also resembled the type B adenocarcinomas, and when again transplanted failed to grow during a 2-month observation period. Transplants of the last type C tumor did not grow while kept under observation for 3.5 months.

The fact that, upon transplantation, 2 of the type C adenocarcinomas produced type B tumors, together with the change of one type B tumor to a predominant type C, suggests that the peculiar adenocarcinoma may be a variant of the type B tumor (which appears especially in old $C \times C_3H$ hybrids), and does not represent a distinct entity.

Most type A and type B adenocarcinomas grew rapidly when transplanted: 6 of the 10 type A tumors and 16 of the 20 type B tumors produced definite growths within 1 month after transplantation.

Of the 21 transplanted tumors which were tested for the presence of the tumor agent and, as stated previously, were the most rapidly growing transplants, 7 were type A and 13 were type B adenocarcinomas; 1 was a primary adenoacanthoma which, upon transplantation, grew as a type B adenocarcinoma. None of the type C adenocarcinomas was tested for the agent.

In addition to the mammary tumors which were taken for section, several myoepitheliomas were also removed. These are subcutaneous tumors arising most often from the parotid salivary gland and located in the cervical region, but they have been found rarely in other subcutaneous areas, where they may originate from mammary-gland elements.

These tumors have been briefly described and photographed previously (*18*). They must always be distinguished from mammary tumors, with which they may be confused because of the subcutaneous location. The cellular arrangement suggests a stratification, and transitions from a cuboidal-cell layer adjacent to vascular spaces to spindle cells which may line indistinct clefts or cystic spaces (fig. 12). With an appropriate stain (phosphotungstic-acid hematoxylin), myofibrils have been demonstrated in association with the spindle cells. On gross examination the cystic centers of these tumors are filled with a mucinous fluid, so they can be tentatively identified at autopsy, but they require microscopic confirmation. Three were found in the 86 strain C mice with the agent, and 4 in the C×C_3H hybrids of the present study. Although we have no exact figures as to incidence, previous observations indicate that they are as frequent in males as in females, and appear to be relatively common in strain C mice and their hybrids.

Several points may be emphasized as a result of this comparative study of strain C mice and hybrids from strain C mothers and C_3H fathers. Tumors of a predominantly acinar structure were more frequent in young mice with the agent. In the C×C_3H hybrids, 15 of 24 tumors appearing before the age of 20 months were of this type (table 3). So there appears to be some correlation between morphology of mammary tumors and the age of the animal. It also appears, as in a previous study (*12*) on dba mice with and without the agent, that a greater variety of mammary tumor forms will emerge in mice in which the agent is absent or weak, Of particular interest in the hybrid mice is a mammary-tumor variant composed of cystic spaces lined by epithelial cells with an outer layer of spindle cells similar to a "precancerous lesion" described by Haaland, which has been seen in a pure form only in old hybrid mice, and is most frequent in hybrids in which a parent is from strain C.

This study also indicates that the morphology of mammary tumors may be somewhat affected by the strain of the tumor-bearing mice. A strain difference cannot be detected unless large groups of tumors are available, for individual tumors may appear identical, in whatever strain they arise, but the unusual forms emerge more frequently in given strains, particularly when the tumor agent is absent from stocks of mice of this strain. More extended studies of tumors from mice of agent-free inbred strains will be required before a definite conclusion on this point is reached, but carcinosarcomas occurred frequently in agent-free dba mice (*12*) treated with methylcholanthrene, whereas no carcinosarcomas appeared in the strain C hybrids of the present study. However, another unusual mammary-tumor variant (adenocarcinoma type C) was frequent in the latter mice.

It may also be true that the tumors appearing in the older mice without the agent offer a closer parallel to human cancer than do tumors appearing in young mice with the agent. An acinar, or lobular pattern, is relatively rare in the human being. Morphologic studies on tumors of the mammary-gland area developing in old mice without the agent may

also furnish a closer parallel to the early studies on the morphology of mammary tumors in mice made before inbreeding had concentrated the tumor agent in certain strains. Such studies are also revealing rare tumors of unusual interest that have not been found in the high-mammary-tumor strains with the tumor agent.

SUMMARY

An experiment was performed to ascertain whether low-mammary-tumor-strain C females harbored a weak or masked mammary tumor-agent.

The C females were exposed to a single dose of 25, 50, 100, or 200 r of whole body X-radiation and then bred to nonirradiated high-mammary-tumor-strain C_3H males. If irradiation enhanced the activity of the agent in C females then the $C \times C_3H$ hybrids born to irradiated females should develop tumors at an earlier average age than those born to nonirradiated C females.

The results confirmed the high incidence of mammary tumors in F_1 hybrids born to strain C females and strain C_3H males, but there was no significant differences between the tumor incidences or the average ages at which tumors arose in the hybrids born to irradiated and nonirradiated C mothers.

Five hybrids, representing 4 litters, developed mammary tumors at an early age and carried the mammary tumor-agent. This also confirmed previous observations. The 5 mice were offspring of C females that had received 100 or 200 r of X-radiation. Litter mates of these hybrids developed tumors late in life, and, apparently, did not carry the agent.

Of the 4 C mothers of agent-bearing hybrids, 2 developed mammary tumors and 2 did not. Their spleens were tested for the agent by inoculation into susceptible test animals and the results indicated that 3 carried the agent and 1 did not.

Four other strain C females developed tumors. Two were exposed to X-radiation and 2 were nonirradiated controls. The spleen of 1 irradiated mouse was tested and found to contain the agent. F_1 hybrid offspring of this mouse developed tumors or died tumor free at a late average age, and, presumably, did not harbor the agent.

The findings in C females extended previous results by showing that, after mating with strain C_3H males, some strain C females possessed the agent and that these mice did not apparently transmit the agent to all their offspring.

Studies on the histologic appearance of the tumors in $C \times C_3H$ hybrids revealed 4 groups of tumors. Three of these groups were adenocarcinomas: In one (type A) the acinar structure predominated, another (type B) showed little acinar formation but possessed a variable pattern of solid groups of epithelial cells, single layers of cuboidal cells lining cystic spaces or growing as papillary projections into cysts, and cords of cells separated by a heavy stroma. In the third (type C) there were many small cysts and epithelial proliferation in the interstitial tissue.

The fourth group of tumors were adenoacanthomas showing large areas of squamous-cell elements.

Type A adenocarcinomas predominated in the 5-to-19-month-old hybrids, and were the only tumors encountered in the 5 young agent-bearing hybrids, whereas type B predominated in hybrids over 19 months of age. Thus, there was a correlation in the morphology of these mammary tumors and the age of the animals in which they arose.

REFERENCES

(1) ANDERVONT, H. B.: Relation of milk influence to mammary tumors of hybrid mice. J. Nat. Cancer Inst. 5: 391–395 (1945).

(2) ANDERVONT, H. B., and DUNN, T. B.: Efforts to detect a mammary-tumor agent in strain C mice. J. Nat. Cancer Inst. 8: 235–240 (1948).

(3) ANDERVONT, H. B.: Incidence of mammary tumors in mice of strain C_3H and in descendants of fostered strain C. J. Nat. Cancer Inst. 10: 193–200 (1949).

(4) ANDERVONT, H. B., and DUNN, T. B.: Mammary tumors in mice presumably free of mammary-tumor agent. J. Nat. Cancer Inst. 8: 227–233 (1948).

(5) ———: Further studies on relation of mammary-tumor agent to mammary tumors of hybrid mice. J. Nat. Cancer Inst. 9: 89–104 (1948).

(6) Biologic effects of long-continued whole body irradiation with gamma rays on mice, guinea pigs, and rabbits. National Nuclear Energy Series, Division IV, vol. 22-b, in press. Wiley Co.

(7) LORENZ, E., HESTON, W. E., and ESCHENBRENNER, A. B.: Effects of long-continued irradiation with gamma rays on mammary tumor incidence in C_3Hb female mice. Quarterly report, May 1948 to Aug. 1948. Argonne National Laboratory. 101–106.

(8) FOULDS, L.: Mammary tumours in hybrid mice: A sex factor in transplantation. Brit. J. Cancer 1: 362–370 (1947).

(9) ———: Mammary tumours in hybrid mice: Hormone responses of transplanted tumours. Brit. J. Cancer 3: 240–246 (1949).

(10) HUMMEL, K. P., LITTLE, C. C., and EDDY, M. S.: Studies on mouse mammary tumor agent. II. Neutralization of agent by placenta. Cancer Research 9: 135–136 (1949).

(11) FOULDS, L.: Mammary tumours in hybrid mice: Presence and transmission of mammary tumor agent. Brit. J. Cancer 3: 230–239 (1949).

(12) ANDERVONT, H. B., and DUNN, T. B.: Response of mammary-tumor-agent free strain dba female mice to percutaneous application of methylcholanthrene. J. Nat. Cancer Inst. 10: 895–926 (1950).

(13) HAALAND, M.: Spontaneous tumours in mice. 4th Sc. Rep. Imp. Cancer Res. Fund 1–13 (1911).

(14) HESTON, W. E., DERINGER, M. K., DUNN, T. B., and LEVILLAIN, W. D.: Factors in the development of spontaneous mammary gland tumors in agent-free strain C_3Hb mice. J. Nat. Cancer Inst. 10: 1139–1155 (1950).

(15) HAALAND, M.: Les Tumeurs de la souris. Ann. de l'Inst. Pasteur 19: 165–207 (1905).

(16) BONSER, G. M.: Miscroscopical study of evolution of mouse mammary cancer, effect of the milk factor and comparison with human disease. J. Path. & Bact. 57: 413–422 (1945).

(17) BASHFORD, A. Behavior of tumour cells during propagation. 4th Sc. Rep., Imp. Cancer Res. Fund. 131 (1911).

(18) LIPPINCOTT, S. W., EDWARDS, J. E., GRADY, H. G., and STEWART, H. L.: Review of some spontaneous neoplasms in mice. J. Nat. Cancer Inst. 3: 199–210 (1942).

PLATE 179

FIGURE 1.—Adenocarcinoma (type A). Reproduction of mammary acini. C×C_3H hybrid, 29 months old. Hematoxylin and eosin. × 235.

FIGURE 2.—Adenocarcinoma (type B). Cells are of large size, they tend to line small cysts and grow as papillary projections. Abundant stroma. C×C_3H hybrid, 27 months old. Hematoxylin and eosin. × 235.

FIGURE 3.—Adenocarcinoma (type B). Large cells growing in cords. Margin shows lymphocytic infiltration. C×C_3H hybrid, 17 months old. Hematoxylin and eosin. × 235.

FIGURE 4.—Adenocarcinoma (type B). Cells growing in solid sheets. C×C_3H hybrid, 25 months old. × 235.

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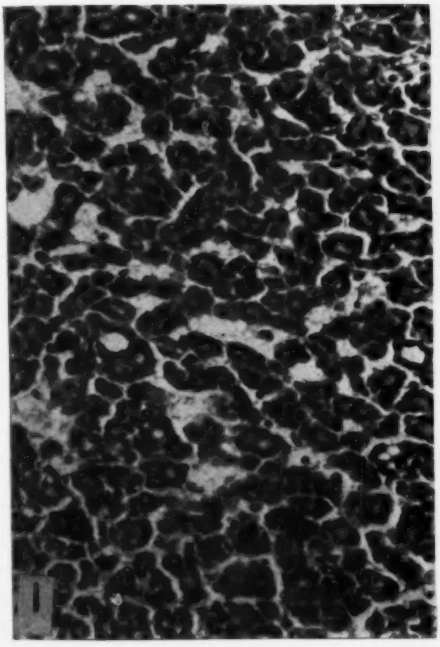
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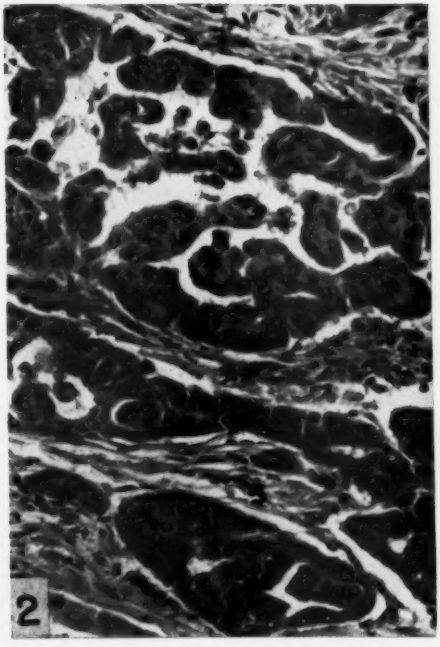
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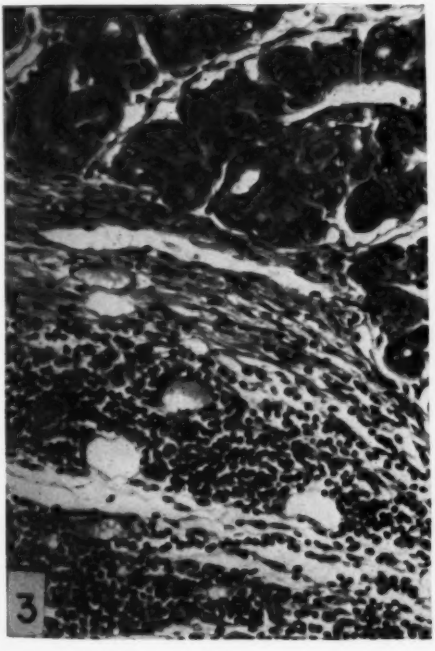
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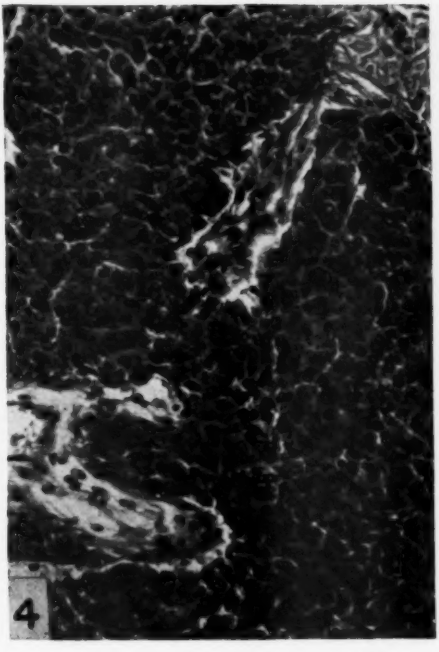
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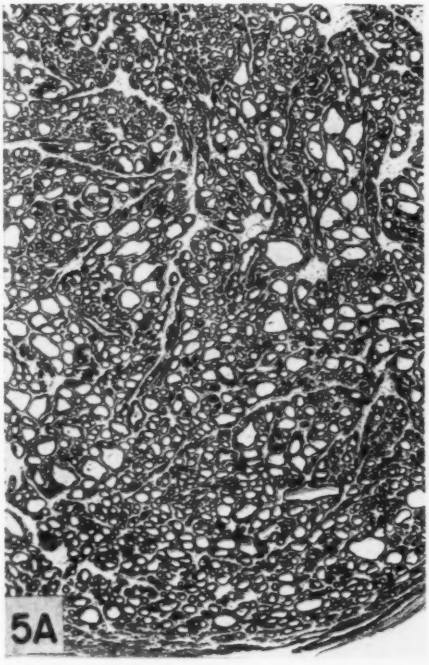
PLATE 180

FIGURE 5.—Adenocarcinoma (type C). Low-power views to show that same pattern of cystic spaces extends through entire tumor. *A*, C×C_3H hybrid, 21 months old. *B*, C×C_3H hybrid, 22 months old. Hematoxylin and eosin. × 20.

FIGURE 6.—Adenocarcinoma (type C). High power to show columnar cells lining cysts, surrounded by layer of spindle cells. *A*, C×C_3H hybrid, 25 months old. *B*, C×C_3H hybrid, 22 months old. (Same tumor as in 5*B*.) Hematoxylin and eosin. × 235.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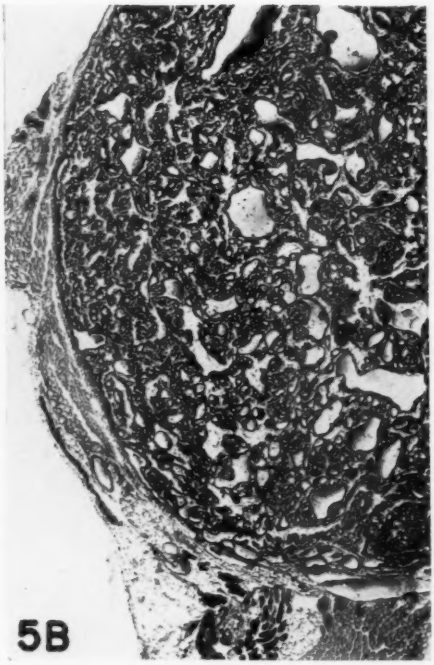
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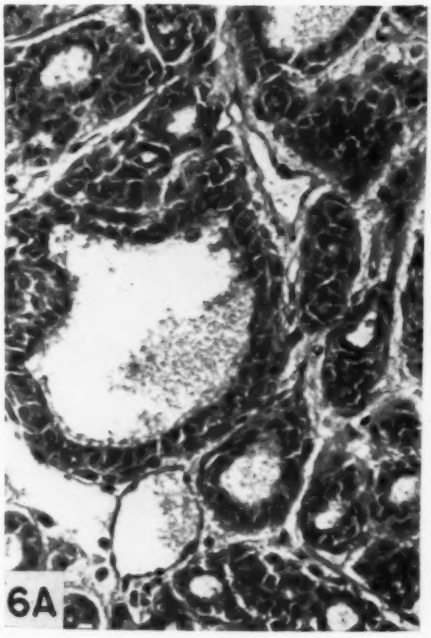
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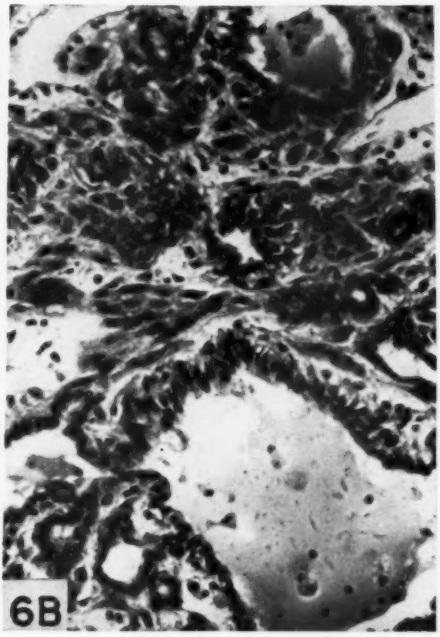
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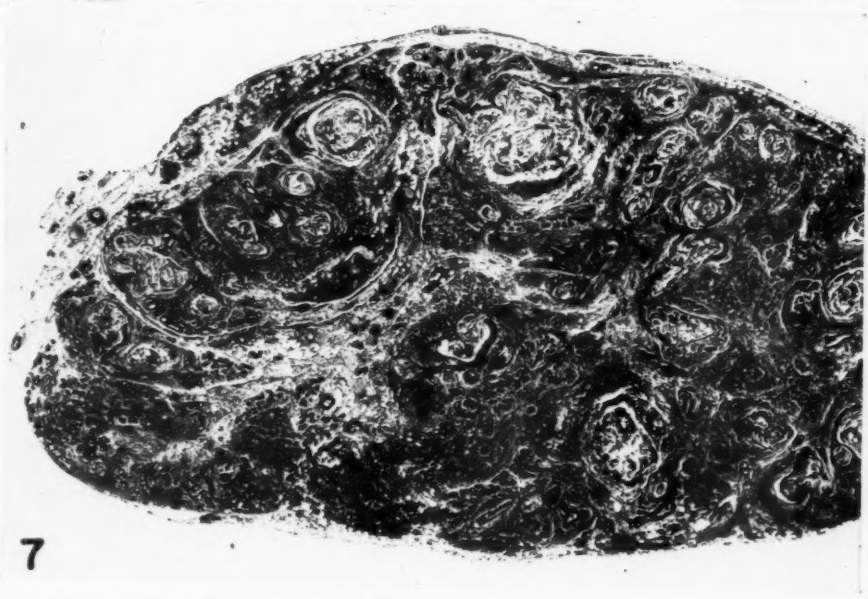
PLATE 181

FIGURE 7.—Adenoacanthoma, with lymphocytic infiltration. C×C_3H hybrid, 26 months old. Hematoxylin and eosin. × 20.

FIGURE 8.—*A*, Carcinoma composed of large detached cells. Cell marked with arrow shows an eosinophilic nuclear inclusion. C×C_3H hybrid, 26 months old. Hematoxylin and eosin. × 235. *B*, Low-power view to show general structure of tumor. Hematoxylin and eosin. × 20.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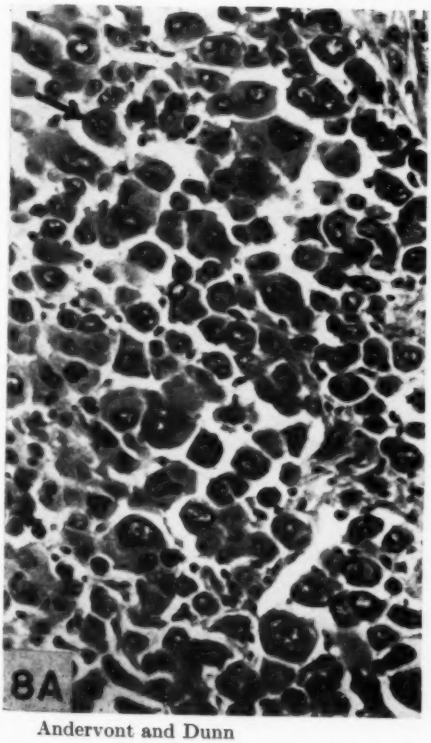
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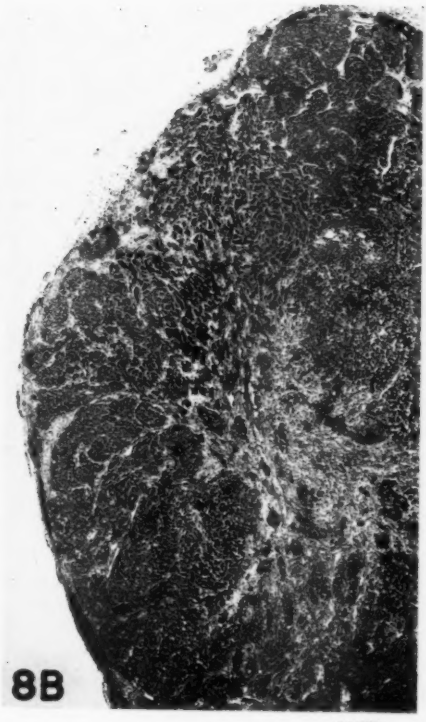
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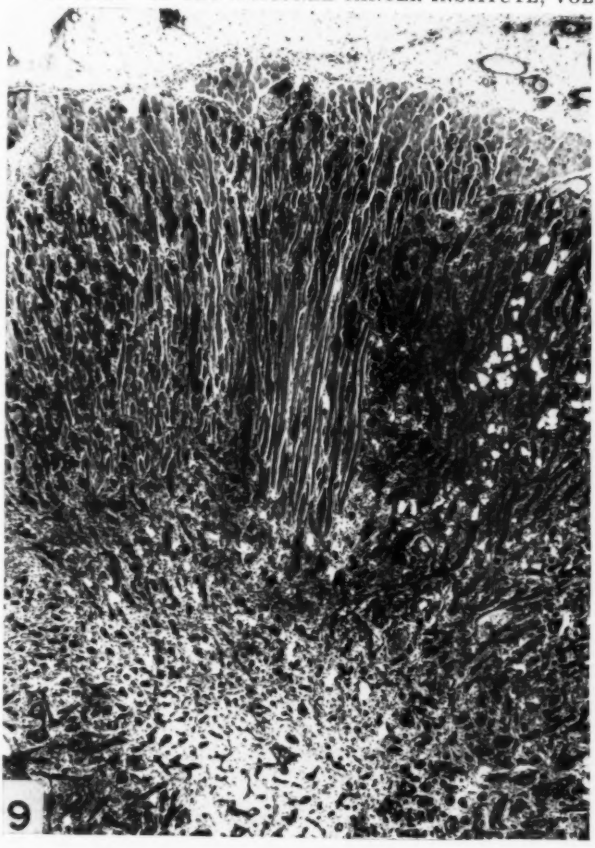
PLATE 182

FIGURE 9.—"Molluscoid" form of tumor. Note infiltration in muscle, yet tumor pattern is still maintained. Strain C, 20 months old. Hematoxylin and eosin. × 40.

FIGURE 10.—Adenocarcinoma showing secretion vacuoles in epithelial cells. Hematoxylin and eosin. × 235.

FIGURE 11.—Adenocarcinoma (type B) showing lymphocytic infiltration at margin. C×C_3H hybrid, 29 months old. × 235.

FIGURE 12.—Myoepithelioma. Note row of cuboidal cells in the center, and transition to a spindle cell. Hematoxylin and eosin. × 235.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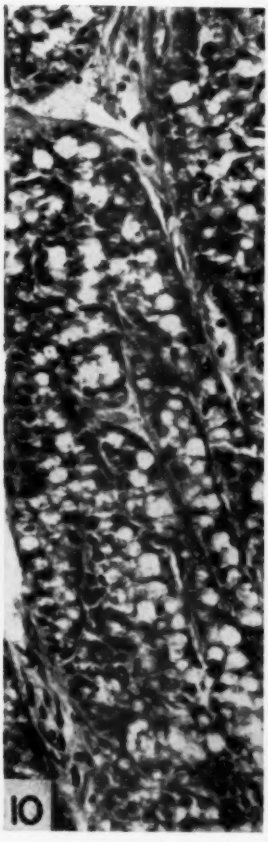
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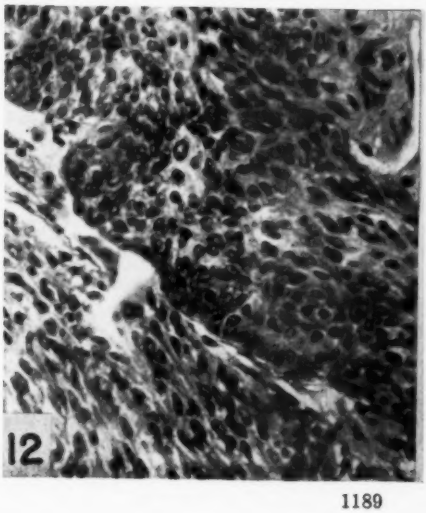
12

STUDIES ON FATTY ACID OXIDATION BY NORMAL AND NEOPLASTIC LIVER [1]

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Investigations of the *in vitro* oxidation of fatty acids by tumors as well as studies of the oxidation of fatty acids by normal tissues have until recently been difficult, because of the relatively low activity of the enzymes involved (as compared, for example, to certain peptidases and glycolytic enzymes), the great lability of these enzyme systems, the lack of specific methods, and particularly the insufficient knowledge concerning the cofactors necessary for activity. The studies of Muñoz and Leloir (*1*) and of Lehninger (*2*) indicated the importance of adenine nucleotides, magnesium ions, inorganic phosphate, and cytochrome in fatty acid oxidation. In addition, Lehninger (*3*) has shown that octanoic acid can be quantitatively oxidized to acetoacetic acid in a washed particle preparation fortified with these cofactors and catalytic amounts of malate. Although the individual steps and the intermediates in fatty acid oxidation are as yet unknown, it is possible to study the over-all conversion of fatty acid to acetoacetic acid in liver preparations, and to carbon dioxide and water in the kidney (*4*). The enzymes involved in fatty acid oxidation appear to be localized in the sedimentable tissue particles (*5*). It is desirable, therefore, in studying this system, to use washed tissue preparations in order to concentrate the activity and to remove tissue components capable of interfering with the determination of fatty acid oxidation. Recent studies (*6*, *7*) indicate that the enzymes involved in the oxidation of fatty acids are associated with the mitochondrial fraction of liver. The present report deals with the oxidation of octanoic and hexanoic acids by normal rat and mouse liver preparations and by hepatomas derived from these tissues.

EXPERIMENTAL

Transplanted spontaneous hepatoma 98/15 (liver-cell carcinoma), originally obtained from Dr. A. B. Eschenbrenner, carried in strain C_3H mice for 56 generations without histologic modification, transplanted hepatoma 112B (liver-cell carcinoma), originally induced by chloroform ingestion (*8*) and obtained from Dr. Eschenbrenner, carried in strain A mice for 36 generations with no histologic change, and N,N-dimethyl-p-

[1] Received for publication January 31, 1950.

aminoazobenzene-induced hepatoma in Osborne-Mendel rats, kindly donated by Dr. J. White, were employed in this study. The rats and mice were fasted for 48 and 24 hours, respectively.

The procedure employed for the determination of fatty acid oxidation was similar to that elaborated by Lehninger (*5*). The animals were sacrificed by decapitation, bled, and the liver or tumor tissue immediately removed and placed in a container chilled in ice. The tissues were then homogenized in two volumes of ice-cold 0.013 M sodium phosphate buffer of pH 7.8, containing 0.13 M potassium chloride, in an all-glass Potter-Elvehjem homogenizer. The homogenate was diluted with an equal volume of this buffer, and centrifuged at 2200 g, at 0° for 3 minutes. The sediment was washed twice with buffer and was usually made up to half the original volume [twice as concentrated as Lehninger's preparation (*5*)]. It was noted that the mitochondrial layer in the sediment was less extensive in the tumor preparations than in those of normal liver. Recent experiments of Drs. G. H. Hogeboom and W. C. Schneider indicate also that the mitochondria of hepatoma 98/15 are deficient in quantity (*9*).

Sodium octanoate and hexanoate solutions were prepared from the corresponding acids [2] which were purified by distillation. L-malic acid [2] was prepared from the racemic compound as described by Dakin (*10*). Cytochrome *c* solution was obtained from the Viobin Corp. and thoroughly dialyzed against distilled water before use. Sodium adenosine triphosphate was obtained from Rohm & Haas Co.

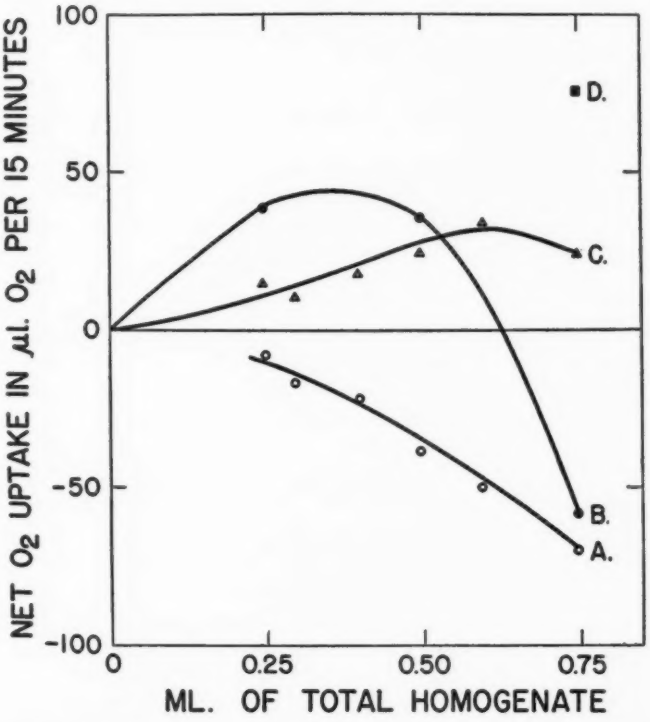
The vessels, containing all additions except enzyme, were chilled at 0° C. Alkali was placed in the center well. Following addition of enzyme the vessels were equilibrated for 5 minutes at 37° C. The taps were then closed and readings taken at 5-minute intervals. Q values were calculated from the linear portion of the oxygen uptake curves (about 15 minutes at 37° C.), and expressed in terms of protein nitrogen concentration.

RESULTS

Under the conditions employed total homogenates were found to be unsatisfactory for comparative oxidation studies because of the wide range of values obtained with normal liver. Considerable variation in activity was observed with homogenates of normal rat liver while the washed particle preparations obtained from the same homogenates gave consistent results (within 10 to 15 percent). Many of the previously reported studies on this system have been carried out with washed particle preparations rather than with homogenates. It seemed of interest, however, to employ homogenates for several exploratory experiments. It was generally found that with a more concentrated homogenate (1 part liver: 2 volumes of buffer), the oxygen uptake value for the homogenate alone was greater than that for homogenate plus substrate. Correction of these values obtained with substrate by subtracting the values

[2] Obtained from Eastman Kodak Co.

for the homogenate alone thus yielded negative values and represented an apparent inhibition (curves A and B, text-fig. 1). This "inhibition"



TEXT-FIGURE 1.—Effect of rat liver homogenate concentration on oxygen uptake. Final concentrations per vessel were: Sodium adenosine triphosphate, 0.0005 M; cytochrome *c*, 0.00001 M; $MgSO_4$, 0.005 M; KCl, 0.0003 M; sodium L-malate, 0.0003 M; sodium phosphate buffer, pH 7.4, 0.01 M. Volume, 2.5 ml.
Curve A: 5 micromoles octanoate and homogenate (1:2).
Curve B: Same as A except with 3 micromoles.
Curve C: 3 micromoles sodium octanoate and homogenate (1:5).
Point D: 3 micromoles octanoate and homogenate (1:9).

could be avoided by reducing the concentration of the homogenate to that of 1 part liver and 5 parts of buffer (curve C, text-fig. 1). Upon further dilution of the homogenate higher corrected values were obtained (*cf.* Point D, text-fig. 1). It would appear that appreciable inhibition of endogenous respiration by fatty acid occurs with more concentrated homogenates.

In contrast to the data obtained with total homogenates, the values obtained with particle preparations were found to be directly proportional to tissue concentration except at very high concentrations. No negative net values were obtained even with particle preparations equivalent in concentration of particles to homogenates prepared with 2 ml. of

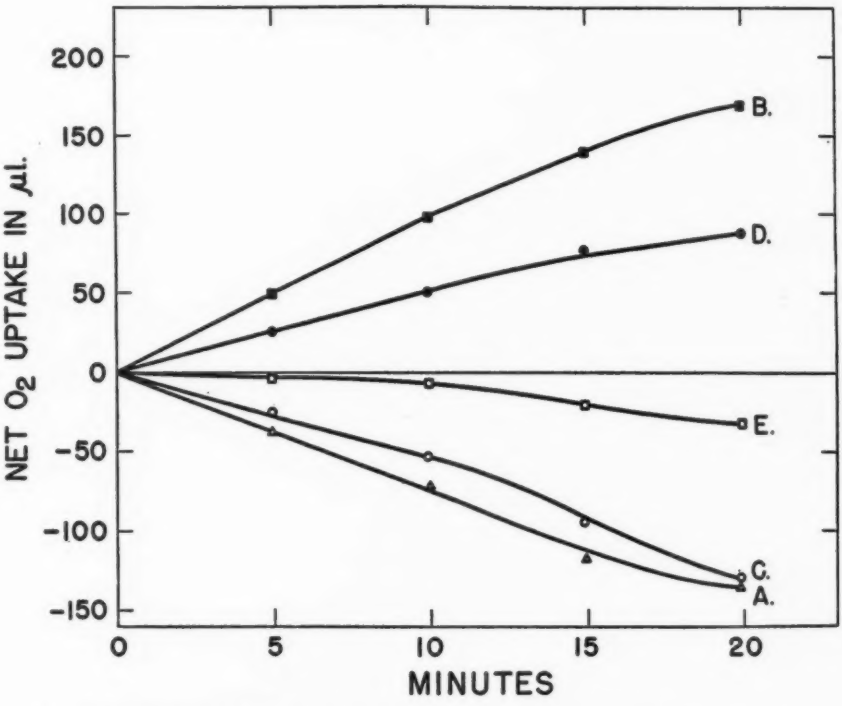
buffer per gram of liver. Although wide variability and negative net manometric values in fatty acid oxidation studies have been reported previously (*2, 11*), it seemed of interest to investigate this finding further. An attempt was made to determine whether the activity of the washed particle preparation could be inhibited by suspension of the particles in the supernate obtained in the first centrifugation (*cf.* procedure described above). Thus, the following fractions were prepared from the same liver:

(1) Whole homogenate.
(2) Washed particle preparation.
(3) Supernate obtained from the first centrifugation of (2).
(4) (3) heated at 100° C. for 3 minutes.

In addition, a supernate similar to fraction No. 3 above was prepared in the same manner a day previous to this experiment and dialyzed against distilled water overnight. All the supernate fractions were inactive. The results obtained with the other fractions or combinations of washed particles and the different supernate fractions, (the tissue concentrations being adjusted so as to be comparable to that of the total homogenate) are given in text-figure 2. Suspension of the washed particles in the supernate No. 3 resulted in a marked inhibition that was not present when the particles were suspended in the heat-treated supernate No. 4. The inhibition was less marked when the particles were suspended in dialyzed supernate. Increasing the concentrations of ATP, cytochrome *c*, or $MgSO_4$ to 5 times those usually employed yielded no change in the inhibition in the case of the total homogenates. Although the explanation of the variability and apparent inhibition is not evident, it would appear that a heat-labile, nondialyzable factor (or factors) may be involved. Further studies of this phenomenon are in progress. Since the use of these homogenates for comparative studies did not appear feasible, at least under these conditions, washed particle preparations were employed for all the comparative work.[3]

The results of the comparative studies are summarized in table 1. Curves describing the time-course of the oxidation of octanoate by various mouse hepatic tissues are given in text-figure 3. From the Q_{O_2} (N)

[3] Because of variable results reported on the effects of malate and malonate (*3, 7*) these compounds were investigated with total homogenates of 1 part liver to 9 parts buffer, and with particle preparations. Employing sodium L-malate, 0.0003 M; potassium malonate, 0.01 M; sodium octanoate, 0.0012 M, the oxygen uptake in microliters per 20 minutes for malate, malonate, both malate and malonate, and in the absence of added malate or malonate, respectively, were: For total homogenate: 180, −3, 8, 77; for the washed particles: 159, 23, 41, 105. It is therefore important to include catalytic amounts of malate in this system [*cf.* (*3*)]. The effects of malate and malonate are more pronounced with total homogenates than with washed particle preparations. Similar results were obtained with more concentrated homogenates.



TEXT-FIGURE 2.—Study of the resolution of the rat liver homogenate.

Experimental details as given in text-figure 1; the sodium octanoate concentration was 5 micromoles; 0.75 ml. of tissue preparation.

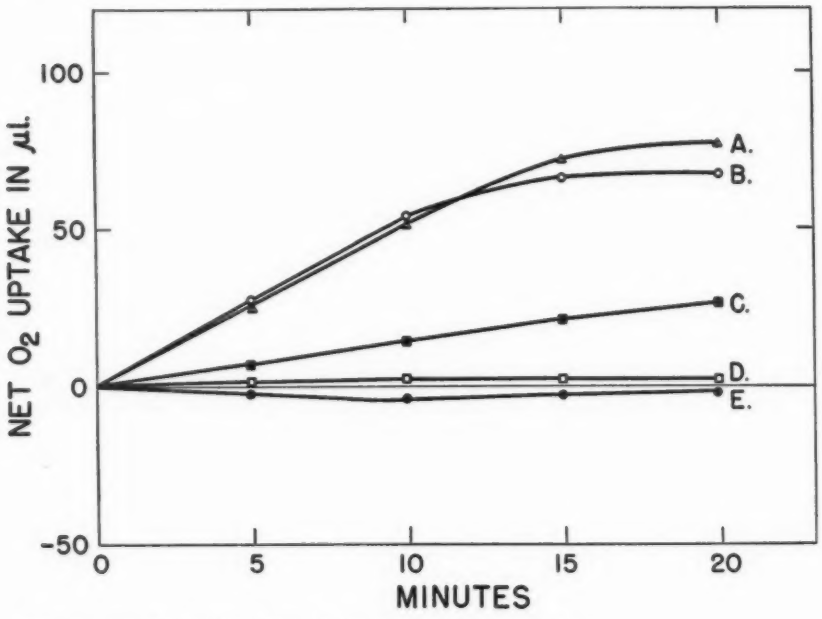
Curve A: Homogenate (1 part liver to 2 parts buffer).

Curve B: Washed particles suspended in buffer.

Curve C: Washed particles suspended in the supernate derived from the first centrifugation in the preparation of washed particles.

Curve D: Washed particles suspended in boiled supernate.

Curve E: Washed particles suspended in dialyzed supernate.



TEXT-FIGURE 3.—Fatty acid oxidation by various mouse tissues.

Final concentrations as given in text-figure 1; 5 micromoles sodium octanoate; 0.75 ml. tissue preparation.

Curve A: Normal liver.
Curve B: Liver of tumor-bearing animal [hepatoma (98/15)].
Curve C: Normal liver plus hepatoma 112B.
Curve D: Hepatoma 112B.
Curve E: Hepatoma 98/15.

values in the table it can be seen that there is a considerably decreased oxygen uptake in the case of the tumors, whether primary or transplanted, and in fetal rat liver. That the azo dye itself affects the fatty acid oxidase system seems unlikely, since the activity of livers of animals fed the dye for several months without development of neoplasms was within normal limits. Particle preparations obtained from mixtures consisting of equal parts of homogenates of normal strain A mouse liver and hepatoma 112B were also studied, and the activity was found to be less than half that of normal liver. Subcutaneous implants of hepatoma 98/15 produced no significant effect on the fatty acid oxidase activity of the liver of the host. Several experiments were also carried out on washed particle preparations obtained from transplanted mouse mammary carcinoma BA[4] and transplanted thymoma.[5] No fatty acid oxidase activity was observed in these studies.

[4] Kindly donated by Dr. M. K. Barrett.
[5] Kindly donated by Dr. A. J. Dalton.

TABLE 1.—*Fatty acid oxidation in normal, neoplastic, and fetal liver* [1]

	Number of animals	$Q_{O_2}(N)$		Protein nitrogen (mgm. per ml.)[2]
		Octanoate	Hexanoate	
MOUSE				
Normal liver	7	85. 7	85. 1	4. 7
Hepatoma 112B	7	6. 7	12. 4	2. 7
Hepatoma 98/15	5	−2. 1	5. 0	1. 8
Normal liver and hepatoma 112B	5	18. 5	17. 3	3. 3
RAT				
Normal liver	4	147.	112.	4. 2
Butter yellow hepatoma	4	−9. 4	4. 3	2. 8
Butter yellow liver	3	137.	77. 0	4. 2
Fetal liver	4	−2. 2	0	2. 5

[1] Conditions as given in text-fig. 1; initial substrate concentration was 5 micromoles; 0.75 ml. of tissue preparation.
[2] Acknowledgment is appreciatively made to Mr. Robert J. Koegel for performing the nitrogen analyses.

DISCUSSION

The extremely low capacity of tumors to oxidize fatty acids is of interest in view of the tendency of many oxidative systems to be decreased in activity in neoplastic tissues (*12*). Failure of tumor slices to oxidize fatty acids has been reported by Ciaranfi (*13*), although unfortunately control tissues were not included in this study. Recently Vestling *et al.* (*14*) reported that livers infiltrated with leukemic cells exhibited a decrease in fatty acid oxidation, usually corresponding to the amount of infiltration. In several cases, however, an appreciable loss of fatty acid oxidase activity was observed in livers that were infiltrated to only a small extent. This phenomenon may be explained on the basis of competition (*14*) or possibly inhibition. A similar mechanism may be considered in the case of the experiments with mixtures of normal mouse liver and mouse hepatoma described above, in which less than the additive oxidation was observed.

Preliminary experiments indicate that octanoate disappears (*15*) in the absence of appreciable oxygen uptake in the case of primary rat hepatoma, suggesting that octanoate may be catabolized *via* an anaerobic mechanism in this tissue, as has been suggested by Weinhouse *et al.* (*16*) in experiments on octanoate disappearance in normal liver slices. Such a pathway might become more significant when the oxidative mechanism for fatty acid catabolism is reduced in activity. Further studies are necessary to evaluate this possibility.

SUMMARY

The oxidation of hexanoic and octanoic acids by washed tissue preparations obtained from homogenates of normal rat and mouse liver, 2 transplanted mouse hepatomas, butter-yellow-induced rat hepatoma, and fetal rat liver, has been studied. The preparations derived from the neoplastic tissues and fetal liver showed a considerably reduced capacity to oxidize fatty acids.

REFERENCES

(*1*) MUÑOZ, J. M., and LELOIR, L. F.: Fatty acid oxidation by liver enzymes. J. Biol. Chem. 147: 355–362 (1943).

(*2*) LEHNINGER, A. L.: Relationship of adenosine polyphosphates to fatty acid oxidation in homogenized liver preparations. J. Biol. Chem. 157: 363–381 (1945).

(*3*) ———: Quantitative study of products of fatty acid oxidation in liver suspensions. J. Biol. Chem. 164: 291–306 (1946).

(*4*) GRAFFLIN, A. L., and GREEN, D. E.: Studies on cyclophorase, II Complete oxidation of fatty acids. J. Biol. Chem. 176: 95–115 (1948).

(*5*) LEHNINGER, A. L., and KENNEDY, E. P.: Requirements of fatty acid oxidase complex of rat liver. J. Biol. Chem. 173: 753–771 (1948).

(*6*) KENNEDY, E. P., and LEHNINGER, A. L.: Intracellular structures and fatty acid oxidase system of rat liver. J. Biol. Chem. 172: 847–848 (1948).

(*7*) SCHNEIDER, W. C.: Intracellular distribution of enzymes. III Oxidation of octanoic acid by rat liver fractions. J. Biol. Chem. 176: 259–266 (1948).

(*8*) ESCHENBRENNER, A. B.: Induction of hepatomas in mice by repeated oral administration of chloroform, with observations on sex differences. J. Nat. Cancer Inst. 5: 251–255 (1945).

(*9*) SCHNEIDER, W. C., and HOGEBOOM, G. H.: Intracellular distribution of enzymes. VI. Distribution of succinoxidase and cytochrome oxidase activities in normal mouse liver and in mouse hepatoma. J. Nat. Cancer Inst. 10: 969–975 (1950).

(*10*) DAKIN, H. D. Resolution of inactive malic acid into optically active forms. J. Biol. Chem. 59: 7–12 (1924).

(*11*) JOWETT, M., and QUASTEL, J. H.: Studies in fat metabolism. II. Oxidation of normal saturated fatty acids in the presence of liver slices. Biochem. J. 29: 2159–2180 (1935).

(*12*) GREENSTEIN, J. P.: Biochemistry of cancer. Academic Press, New York, 1947.

(*13*) CIARANFI, E.: On ability of tumor tissue to oxidize fatty acids in vitro. Am. J. Cancer 32: 561–564 (1938).

(*14*) VESTLING, C. S., WILLIAM, J. N., JR., KAUFMAN, S., MAXWELL, R. E., and QUASTLER, H.: Oxidation of octanoate by liver homogenates from leukemic mice. Cancer Research 9: 639–644 (1949).

(*15*) LEHNINGER, A. L., and SMITH, S. W.: Rapid determination of n-octanoic acid. J. Biol. Chem. 173: 773–783 (1948).

(*16*) WEINHOUSE, S., MEDES, G., and FLOYD, N.: Fatty acid metabolism. Mechanism of ketone body synthesis from fatty acids with isotopic carbon as tracer. J. Biol. Chem. 154: 143–151 (1944).

COMPARATIVE STUDIES OF LIVER GLUCURONIDASE ACTIVITY IN INBRED MICE [1]

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In previous studies of inbred mice (*1*) the liver glucuronidase activity of C_3H (Heston) mice was observed to be 80–90 percent lower than the activity in four other strains. This report presents our observations on a total of 419 animals of eight strains, including three sublines of the C_3H strain. Determinations of kidney and spleen glucuronidase were performed in strains with high and low liver glucuronidase activity. The pH of maximum activity was determined for liver glucuronidase from the A and C_3H strains.

MATERIALS AND METHODS

Inbred mice were obtained from various laboratories as indicated in table 1. Animals were segregated according to sex and maintained in our laboratory for a minimum of five days prior to study. The mice selected for assay weighed 17–25 grams and were of various ages. At the time of autopsy none was found to have a tumor.

The glucuronidase determinations were performed on uncentrifuged tissue homogenates by a modification of the method of Talalay (*2*), employing phenolphthalein glucuronide as the substrate. The details of the procedure have been previously reported (*1*). Glucuronidase activity was expressed as micrograms of phenolphthalein liberated per mg. of moist tissue after incubation for 1 hour with 0.0005 M phenolphthalein glucuronide in acetate buffer, pH 4.5.

RESULTS AND DISCUSSION

The livers of 419 animals of 8 strains were assayed for glucuronidase activity. Three sublines of strain C_3H and 2 of strain CBA were included. The results, combined with those values previously reported (*1*), are summarized in table 1. The liver glucuronidase activity was low in all sublines of the C_3H strain and in the Andervont subline of strain CBA. The mean values for animals of these sublines were 0.19,

[1] Received for publication February 10, 1950.

TABLE 1.—*Liver glucuronidase activity in inbred mice*

Strain and source	Number of animals assayed		Liver glucuronidase activity [1]			
	Males	Females	Mean males	Mean females	Mean group	Standard error of mean
C_3H (Heston)[2 3 4]	80	71	0. 47	0. 41	0. 44	±0. 01
C_3H (Bittner)[5 6]	25	23	. 19	. 19	. 19	±. 01
C_3H (Andervont)[7]	5	5	. 31	. 36	. 33	±. 02
A (Heston)	39	40	3. 7	3. 4	3. 5	±. 06
A (Bittner)	0	7		4. 3	4. 3	±. 28
CBA (Andervont)[8]	5	5	. 26	. 31	. 28	±. 02
CBA (Strong)[9]	4	5	3. 5	4. 2	3. 9	±. 28
B alb C (Heston)	16	15	4. 2	3. 8	4. 0	±. 15
dba (Heston)	20	20	4. 4	3. 7	4. 0	±. 15
RIII (Andervont)	4	5	4. 5	3. 9	4. 2	±. 02
I (Andervont)	5	5	4. 9	3. 8	4. 4	±. 21
C57 black (Heston)	7	8	3. 8	4. 5	4. 2	±. 17

[1] Expressed as micrograms of phenolphthalein liberated per mg. of moist liver after incubation for 1 hour at 37.5° C, pH 4.5.
[2] Separated from Strong's original strain since 1930.
[3] 21 animals of this group did not have the milk factor. Their liver glucuronidase activity averaged 0.51.
[4] In addition 3 animals were found having high activity. All were males. The values were 3.0, 2.5, and 3.1.
[5] Separated from Strong's original strain since 1931, after 22 generations.
[6] 18 animals did not have the milk factor. Their liver glucuronidase activity averaged 0.21.
[7] Separated from Strong's strain by 50-60 generations of inbreeding. Lines separated in 1930.
[8] Separated from Strong's original strain in 1947. The animals assayed were of the F6 generation in Andervont's laboratory.
[9] Strong's original line, inbred since 1920.

0.33, 0.44, and 0.28. Activity in 6 other strains and the Strong subline of strain CBA was 8 to 10 times greater, the mean values ranging from 3.5 to 4.40. The presence or absence of the mammary tumor inciter (milk factor) in C_3H (Heston) and C_3H (Bittner) mice did not alter the liver glucuronidase activity.

These glucuronidase assays were based upon the moist weight of a liver sample, and it seemed possible that differences in water content of the livers might account for the high and low activity observed in the various strains. Accordingly, 10 A and 10 C_3H mice of the Heston sublines were selected for assays based on the dry weight and total nitrogen of the liver sample as well as its moist weight. The results are presented in table 2, where the glucuronidase activity is expressed as (1) micrograms of phenolphthalein liberated per mg. of moist liver, (2) micrograms liberated per mg. of dry weight, and (3) micrograms liberated per mg. of total nitrogen.

On the basis of moist weight the average value for the A mice was 5.5 times that of the C_3H mice. On the basis of dry weight of liver the ratio was 5.2:1 and for total nitrogen 5.9:1. The differences in water content and total nitrogen were not sufficient to account for the different liver glucuronidase activity of the two strains.

The livers of the C_3H and Andervont CBA mice may show low glucuronidase activity if the enzyme in these animals differs from that of the other strains. Two glucuronidase fractions with pH optima of 4.5 and 5.2 were demonstrated by Mills (*4*) in extracts of ox spleen. Kerr (*5*) noted similar fractions in extracts of mouse kidney and liver (strain of mice not specified). In the assay procedure ordinarily employed, incubation is

TABLE 2.—*Glucuronidase activity of A and C_3H livers expressed as a function of moist weight, dry weight, and total nitrogen* [1]

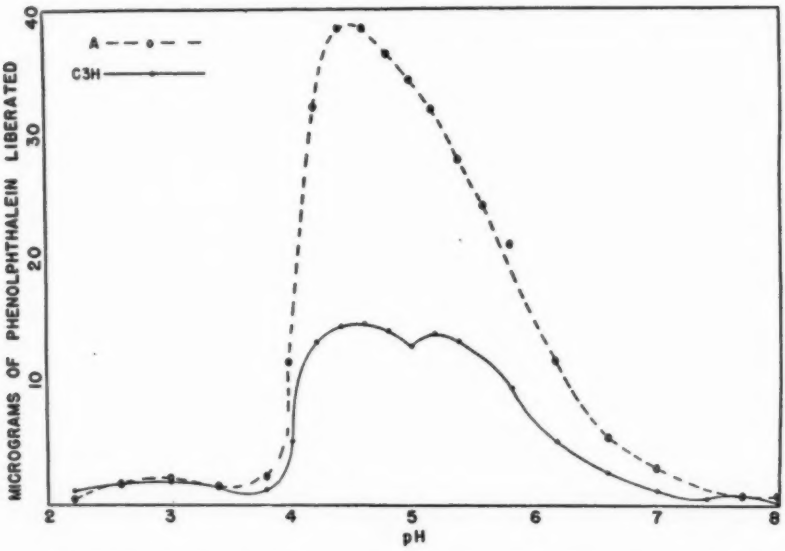
Strain and sex	Moist weight of liver homogenized in 5.0 ml. (mg.)	Total N per 0.5 ml. of homogenate (mg.)	Percent dry weight	Liver glucuronidase activity		
				γ lib. per mg. moist weight	γ lib. per mg. dry weight	γ lib. per mg. total N
C_3H female	297. 0	0. 82	28. 0	0. 63	2. 2	23
Do	286. 2		28. 6	. 59	2. 1	
Do	294. 2	. 79	28. 0	. 66	2. 4	25
Do	319. 4	. 82	28. 6	. 58	2. 0	23
Do	304. 0	. 84	29. 9	. 49	1. 6	18
C_3H male	315. 6	. 98	30. 0	. 61	2. 0	20
Do	293. 8	. 78	29. 7	. 59	2. 0	22
Do	296. 0	. 89	29. 8	. 71	2. 4	24
Do	314. 2	1. 00	29. 8	. 62	2. 1	19
Do	309. 6	. 95	29. 6	. 67	2. 2	22
Mean for C_3H mice			29. 2	. 62	2. 1	22
A female	99. 2	. 26	32. 4	3. 6	11. 0	136
Do	95. 4	. 25	32. 0	3. 3	10. 3	125
Do	105. 8	. 31	31. 8	3. 5	10. 9	119
Do	96. 4	. 25	31. 5	3. 2	10. 0	122
Do	108. 2	. 26	32. 4	2. 6	8. 1	109
A male	98. 0	. 26	31. 3	3. 9	12. 3	126
Do	89. 0	. 25	31. 4	3. 7	12. 3	133
Do	100. 0	. 26	31. 2	3. 5	11. 3	135
Do	98. 2	. 24	30. 8	3. 6	11. 5	145
Do	100. 6	. 26	31. 6	3. 5	11. 0	134
Mean for A mice			31. 5	3. 4	10. 9	128

[1] Each animal was killed and two portions of liver removed and immediately weighed on a micro-torsion balance. One portion was homogenized in 5.0 ml. of water and 0.5 ml. aliquots of this homogenate assayed in duplicate for glucuronidase activity. 100 mg. of A liver liberated sufficient phenolphthalein for precise measurement but 300 mg. of C_3H liver were required. A 2.5 ml. portion of the homogenate was assayed for total nitrogen by a modification of Ma's method (3). The second portion of liver (400–500 mg.) was dried to constant weight at 105° C. From these data the percent of solids and total nitrogen in the homogenate was calculated.

carried out at pH 4.5. If the glucuronidase of C_3H and Andervont CBA livers was most active at a pH other than 4.5 low values would be obtained.

Experiments were performed relating the glucuronidase activity of A and C_3H liver homogenates to pH. Typical curves are shown in text-figure 1. The enzyme from the A strain was most active at pH 4.4–4.6. When C_3H liver homogenates were assayed, maximum activity also occurred at pH 4.4–4.6 and a second peak of lesser magnitude was seen at pH 5.2. The two peaks correspond to those described by Mills and Kerr. The activity of the C_3H homogenates at both pH 4.6 and 5.2 was low compared to the activity of A liver homogenates at pH 4.6. (See footnote to text-fig. 1.) Two glucuronidase fractions were not demonstrated in homogenates of A liver.

Determinations of spleen, kidney, and liver glucuronidase activity were performed in mice of the CBA (Andervont), C_3H and A strains. These values are given in table 3. Activity in the kidneys and livers of the C_3H and CBA mice was 10–20 percent of that in the A mice. The activity in C_3H and CBA spleens was 50–60 percent of that in the A



TEXT-FIGURE 1.—pH-Activity curves for liver glucuronidase from the A and C_3H strains.[1]

[1] 1319 mg. of C_3H liver and 493 mg. of A liver were each homogenized in 45.0 ml. of water. 0.5 mg. aliquots of these homogenates were assayed in duplicate. Digestion mixtures consisted of 4.0 ml. of 0.1 M buffer, 0.5 ml. of 0.005 M phenolphthalein glucuronide, and 0.5 ml. of homogenate. Buffers of pH 2.2-3.8 were phthalate-HCl mixtures; 4.0-6.2 were phthalate-NaOH mixtures; 6.6-8.0 KH_2PO_4-NaOH mixtures. The standard phenolphthalein curve was constructed at pH 10.0 but the pH of the mixtures after the addition of glycine buffer varied from 9.7 to 10.2. This was not adjusted and resulted in slightly increased slope of the curves from pH 2.2 to 4.6 and decreased slope from pH 4.6 to 8.0.

spleens. It was noted that the glucuronidase activity of the C_3H spleen was considerably higher (1.6) than that of C_3H liver (0.36).

The incidence of spontaneous hepatoma in the males of the CBA (Andervont) strain is approximately 30 percent and in C_3H (Heston) mice is 25 percent (*6*).[2] In the females of these strains hepatomas occur in 6–10 percent. In certain strains with high liver glucuronidase activity the incidence is less than 1 percent (*1*). This possible relationship between low glucuronidase activity and hepatoma formation is presently being investigated. However, Strong (*7*) has reported the frequent occurrence of spontaneous hepatomas in his CBA mice, and the livers of these animals show high glucuronidase activity. The Andervont and Strong sublines of the CBA strain are separated by only six generations and both are closely related to the C_3H strain. The different glucuronidase activity in the two sublines of the CBA strain is unexplained.

SUMMARY

Glucuronidase activity was assayed in the livers of 419 mice of 8 inbred strains. Activity was low in all C_3H sublines and in the Andervont subline of the CBA strain. Liver glucuronidase activity in 6 other

TABLE 3.—*Liver, spleen, and kidney glucuronidase activity of A, C_3H, and CBA female mice*

Strain and source	Glucuronidase activity [1]		
	Liver	Spleen	Kidney
A (Heston)	4. 1	3. 3	1. 5
Do	3. 5	3. 3	1. 5
Do	3. 3	3. 7	1. 9
Do	3. 2	4. 4	1. 9
Do	3. 3	3. 3	1. 7
Mean	3. 5	3. 6	1. 7
C_3H (Andervont)	. 38	1. 6	. 24
Do	. 34	1. 7	. 20
Do	. 41	1. 6	. 23
Do	. 36	1. 6	. 24
Do	. 31	1. 6	. 30
Mean	. 36	1. 6	. 24
CBA (Andervont)	. 33	1. 3	. 28
Do	. 26	1. 4	. 25
Do	. 33	1. 2	. 32
Do	. 30	1. 2	. 27
Do	. 31	1. 2	. 23
Mean	. 30	1. 3	. 27

[1] Expressed as micrograms of phenolphthalein liberated per mg. of moist tissue after incubation for one hour at 37.5° C, pH 4.5.

[3] Personal communication, Dr. H. B. Andervont, National Cancer Institute.

strains and sublines was five times greater. In the C_3H and CBA strains low liver glucuronidase activity was accompanied by low activity in the kidneys and spleen. Detailed assays on A and C_3H mice showed that the difference observed was not due to the water or total nitrogen content of the livers. The pH of maximum activity was the same for liver glucuronidase of the A and C_3H strains.

REFERENCES

(*1*) MORROW, A. G., GREENSPAN, E. M., and CARROLL, D. M.: Liver glucuronidase activity of inbred mouse strains. J. Nat. Cancer Inst. 10: 657–662 (1949).

(*2*) TALALAY, P., FISHMAN, W. H., and HUGGINS, C.: Chromogenic substrates. II. Phenolphthalein glucuronic acid as substrate for the assay of glucuronidase activity. J. Biol. Chem. 166: 757–772 (1946).

(*3*) MA, T. S. and ZUAZAGA, G.: Micro-Kjeldahl determination of nitrogen. Indust. & Engin. Chem. (Analyt. Ed.) 14: 280–282 (1942).

(*4*) MILLS, G. T., Beta-glucuronidase activity of ox spleen. Nature 160: 638 (1947).

(*5*) KERR, L. M. H., CAMPBELL, J. G., and LEVVY, G. A.: Beta-glucuronidase as an index of growth in the uterus and other organs. Biochem. J. 44: 487–494 (1949).

(*6*) ANDERVONT, H. B. and MCELENEY, W. J.: Spontaneous tumors in subline of strain C_3H mice. J. Nat. Cancer Inst. 1: 737–744 (1941).

(*7*) STRONG, L. C., and SMITH, G. M.: Benign hepatomas in mice of CBA strain, Am. J. Cancer 27: 279–284 (1936).

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